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C. F. A. PANTIN, Sc.D., F.R.S.

JOHN R. BAKER, D.Sc.



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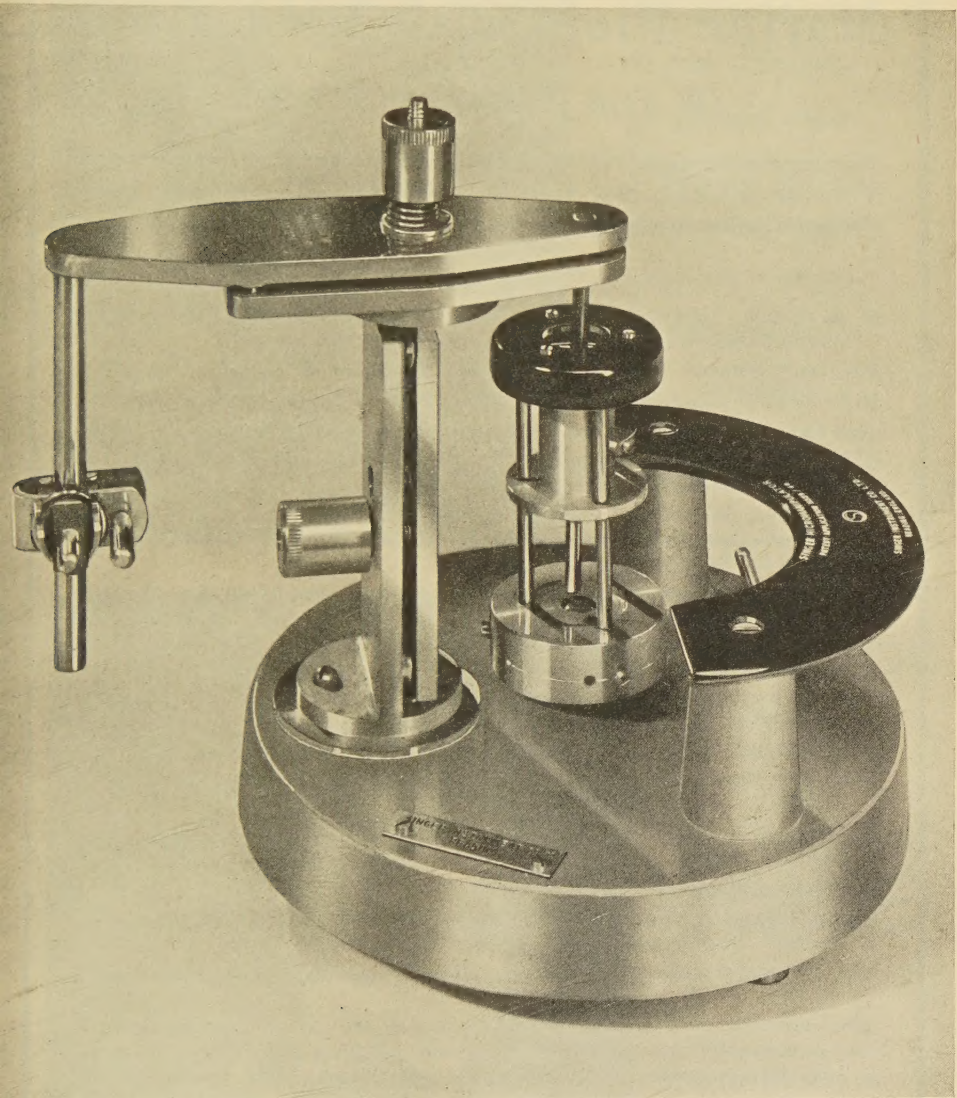
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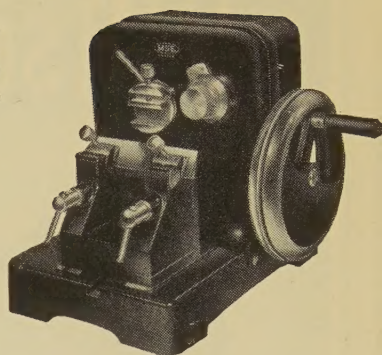
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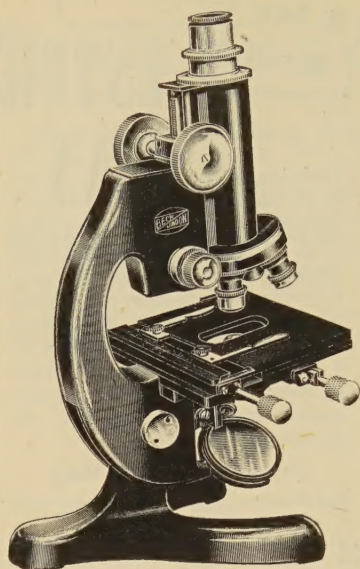
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The Influence of a Gonadotrophin on the Seasonal Changes in the Testis and Deferent Duct of the Chaffinch (*Fringilla coelebs*)

BY

J. W. SLUITER AND G. J. VAN OORDT

(From the Dept. of Endocrinology, Zoological Laboratory, University of Utrecht)

With 3 Plates

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INTRODUCTION

TO investigate the localization of the endocrine function of the testis two methods may be applied:

- (a) activation of the immature gonad by administration of gonadotrophins in juvenile animals;
- (b) activation of the quiescent gonad by administration of gonadotrophins in adult animals possessing a sexual cycle.

In a recent investigation (Sluiter and van Oordt, 1947) the first method was applied and domestic cockerels were used. In this paper it was proved that the classical Leydig cells of the intertubular testis-tissue, called lipoid cells by us, do not secrete the male sex hormone, but that this hormone is produced by the so-called secretion cells, also present in the intertubular tissue.

Then the question arose whether this also applies to adult wild birds with a distinct sexual cycle; so we used the second method mentioned above: adult male chaffinches were treated in different seasons with a gonadotrophin, and the histological and cytological changes induced by this hormone were studied. Just as in the experiments with cockerels gestyl was administered, a gonadotrophin prepared from pregnant mare serum. Contrary to its effect in cockerels it has a definite influence on the generative testis-tissue in

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chaffinches; in this paper therefore the hormonal influence of gestyl on both intertubular tissue and testis-tubules will be considered.

MATERIAL AND METHOD

The material consisted of 11 adult male chaffinches (*Fringilla coelebs*), which had been caught at Loosduinen near The Hague during their autumn migration. They were kept in a large aviary, placed near the window of one of the laboratory rooms. Here they showed all characteristics of the natural sexual cycle. Five birds received gestyl injections in different months of the year. Each experimental bird received 5 doses of 100 I.U. every other day, i.e. 500 I.U. in total, and was autopsied 2 days after the last injection. Two of the experimental birds were killed on 30 November 1943, one on 28 January 1944, one on 5 May 1944, and one on 11 August 1944. The testes were measured immediately *post mortem*. Parts of the same testis were fixed in formalin for general staining with haemalum and eosin or for staining with Sudan III to demonstrate lipoids and with Schultz's fluid to establish the presence of cholesterol. Others were fixed in Champy's fluid for staining cytological details such as mitochondria and granules with the aid of Altmann's acid fuchsin and brilliant-cresylblue. The thickness of the sections was 2–3 μ after Champy-fixation and 7–12 μ in all other cases. In addition, a part of about 3 mm. length was cut out of each left deferent duct at the level of the posterior end of the left kidney, fixed in formalin for general staining, and cut into sections, 7 μ thick.

RESULTS OF GESTYL-ADMINISTRATION

1. *In the Quiescent Stage*

As in other passerine birds the testes of the chaffinch already show a distinct regression just after the breeding season: then they decrease enormously in size, whereas the sperm-cells and spermatogenous cells degenerate for the greater part. In August a complete resting stage is reached, which passes over into the progressive stage towards the end of January.

In this paragraph the results will be described, obtained from 4 control birds and from 3 experimental birds, whose testes were quiescent at the beginning of the experiment. When compared among themselves neither the testes of the control birds nor those of the experimentals showed essential differences.

The macroscopical effect of the hormonal injection on the testis size was very distinct. For convenience' sake we shall indicate the testis size only by one figure: the average size in mm. of the long and short axes of both testes. In control birds the size of the testis in the quiescent stage is about 1 mm.; in birds treated with gestyl in the same stage the testes are much larger and have a size of 2.25–4.25 mm. Consequently the question arises which part of the testis is responsible for this enlargement. Microscopical investigation of the testes showed the following details:

(a) *Testis-tubules*. The testis-tubules of *control birds* have a markedly small diameter, and mostly a minute lumen; they contain, in cross-sections, one or two basal rows of nuclei (Pl. I a). Most nuclei are small and oblong; the others are large, round, and inflated, and are surrounded by a distinct, round cell-body; they represent typical gonocytes. The other cells possess indistinct cell-contours. One gets the impression that these cells have a long cylindrical shape; their apices reach the centre of the testis-tubule, whereas their nuclei lie at their bases.

In preparations stained with Sudan III the central part of the testis-tubules is filled with large lipid globules (Pl. I b). Moreover, Champy-preparations stained with acid fuchsin show that the cylindrical cells, mentioned above, are filled with vacuoles, surrounded by numerous mitochondria (Pl. I c). The contents of these vacuoles have obviously been dissolved in Canada-balsam, for in fresh preparations they are visible as an osmiophil black substance, identical with the sudanophil, orange-coloured lipoids (cf. Pl. I b). Consequently these lipoids must be stored intracellularly, a fact already mentioned by Loisel (1903) for the winter testis of the sparrow.

We are inclined to suppose that these lipoids are the last remains of the fatty degeneration of the previous generation of spermatogenic cells; they must first have been present in the lumen of the testis-tubules, from which they have been resorbed later on by the cylindrical cells, mentioned above.

According to Rowan (1929) in juncos (*Junco hyemalis*) the gonocytes with their large inflated nuclei are destined to degenerate before long. Loisel (1901) is of opinion that in sparrows the cells with small nuclei are the cells from which the next spermatogenesis starts. According to Rowan (1929) and Bullough (1942) the same holds good for the junco and the starling respectively. As we have investigated only a small number of chaffinches, we could not decide this question.

In the *experimental birds* the testis-tubules possess a quite different structure. Their diameter is much larger, which is the principal cause of the considerable testis-enlargement (cf. p. 2 for measurements). This increase in size is accomplished partly by the formation of a lumen in the tubules, partly by a prominent thickening of their walls. As Pl. I a₁ shows, the latter phenomenon must not primarily be ascribed to cell-divisions but to a noteworthy inflation of the cell-protoplasm. It is true, cell-divisions have taken place, but only for the purpose of maintaining a single row of nuclei at the periphery of the tubules.

Contrary to the nuclei in the testis-tubules of the control birds, the nuclei in the tubules of the experimental birds have all become round and inflated; therefore a distinction between gonocytes and cells with small nuclei is impossible here. Meiotic figures (Pl. I a₁) are very scarce or lacking.

The cell-plasma possesses a foamy structure and contains many large vacuoles. After Sudan III staining several lipid-globules are visible (Pl. I b₁). In Champy-preparations of the experimental testes (Pl. I c₁), which

are quite different from those of control testes (cf. Pl. I c), the mitochondria have spread and the compact accumulation of lipid vacuoles does not exist any longer.

From the above it follows that the testis-tubules of chaffinches, treated with gestyl during the quiescent stage, increase distinctly in diameter. The histological structure of these tubules, however, does not show any likeness to that of testes with a beginning spermatogenesis. As the number of control birds is small, we are not sure whether this structure occurs in normal chaffinches or not, but this seems improbable, as it has not been observed in related bird-species, viz. sparrows (Loisel, 1901, 1903), juncos (Rowan, 1929), starlings (Bullough, 1942), and greenfinches (Damsté, 1947).

(b) *Intertubular Tissue and Deferent Ducts.* The histological changes in the intertubular tissue, brought about after gestyl-administration in chaffinches during the quiescent stage, are still more conspicuous.

In the chaffinch, as well as in other birds, the intertubular tissue of the *quiescent testis* is composed almost exclusively of packed masses of small oval nuclei, mostly called connective tissue nuclei in other bird-species (Benoit, 1923a; Rowan, 1929; Bullough, 1942; Damsté, 1947). The testis-tubules are enveloped by these masses, which are separated from each other by a narrow layer of connective tissue cells and blood-vessels (Pl. I c and I d). Though most of the cells belonging to the masses surrounding the testis-tubules probably pass over into differentiated connective tissue cells, we prefer to call them 'indifferent cells' in this stage of development, as then true differentiated connective tissue cells are also present.

Neither lipid (= Leydig) cells nor secretion cells can be found in the intertubular tissue of quiescent testes of control chaffinches. The fact that lipid cells are lacking follows also from the observation that the sudanophil globules (cf. Pl. I b) are small, diffusely spread in this tissue, and obviously lying in the indifferent cells.

In the testes of chaffinches, treated with gestyl during the resting stage, the intertubular tissue has a quite different structure. In the first place its quantity has increased (Pl. I d₁; Pl I d and d₁ are comparable, as the radius of each semicircle is proportionate to the average sizes of the long and short axes of both testes). Moreover, contrary to the intertubular tissue of the control testes, the intertubular tissue of the testes of the experimental birds consists of differentiated connective tissue cells, between which groups of true interstitial cells are found, lacking in control testes. Some of these cells are lipid cells, which follows from the concentration of the sudanophil elements (cf. Pl. I b₁), but for the greater part they are true secretion cells, characterized by many fuchsinophil elements and the lack of lipid vacuoles (cf. Pl. I c₁).

That a great quantity of androgenic substances is produced by this large number of secretion cells may be derived from the fact that the diameter of the deferent ducts of the experimental birds (Pl. I e₁) is much larger than that of the controls (Pl. I e): in the course of 10 days the very narrow

deferent duct of the quiescent bird nearly reached the normal diameter of the reproductive season (Pl. III *e*).

Finally we have to answer the question whether during the normal sexual cycle of the chaffinch an intertubular tissue of similar appearance develops.

After having studied the testes of our control birds, we came to the conclusion that such a large number of secretion cells is never found during the normal sexual cycle of the testis. Normally the increase in diameter of the deferent ducts from the quiescent winter-condition to the functional spring-condition takes more than 10 days, the period of increase in our experimental birds. So it is easily understood that under artificial conditions the abnormally quick increase in diameter of the deferent duct only takes place under the influence of an abnormally high level of male sex hormone, secreted by an excess of secretion cells.

From the above it follows that after gonadotrophin-administration not only an increase in the quantity of inter-tubular tissue is induced, but also its differentiation. Indifferent cells develop into differentiated connective tissue cells, and into special interstitial cells, of which lipid and secretion cells may be distinguished. As, moreover, the male genital ducts react, these secretion cells must have secreted androgen. We came to the same conclusion in our paper on the reaction of the intertubular tissue to gestyl in cockerels (Sluiter and van Oordt, 1947).

2. In the Progressive Stage

At the end of January the first changes in the testes of our control birds were visible. Therefore we chose this time of the year for gestyl-administration. Macroscopically the influence of the hormone on the testes was distinct: the testes measured 3 mm., the control testes only 1.5 mm. As to the size there was no great difference between the testes of January and of August or November control birds. Microscopical investigation, however, showed that the testes of the January control birds possessed a distinctly different structure.

(a) *Testis-tubules.* In the *control bird* the diameter of the testis-tubules, in which a lumen is scarcely visible, is still small; but the wall of these tubules contains several layers of nuclei of almost the same size (Pl. II *c*). Some of these nuclei are surrounded by distinctly limited round cell-bodies, and therefore strongly resemble gonocytes; most cells, however, form a syncytium, containing many minute, dispersed mitochondria. The lipid globules, so characteristic for the tubules of the resting stage, are much reduced in size and number (Pl. II *b*).

In chaffinches *injected with gestyl* in January, the testis-tubules on the whole show the same characteristics as testis-tubules of birds injected in August and November. Only the quantity of lipoids (Pl. II *b*₁) is smaller, which is, however, a matter of course, as the quantity of lipoids has also decreased in the testis-tubules of control birds.

Therefore testis-tubules of birds injected with gestyl in the beginning of

the progressive stage react in the same way as tubules of birds treated during the quiescent stage.

(b) *Intertubular Tissue and Deferent Ducts.* The histology of the intertubular tissue of a *control bird* of January (Pl. II *d*) is totally different from that of the control birds of August and November (Pl. I *d*). The main part of this tissue consists of differentiated connective tissue cells, whereas the indifferent cells only form a narrow layer around the testis-tubules. In the connective tissue groups of special interstitial cells are to be found. In these respects there is much resemblance to the intertubular testis-tissue of chaffinches treated with gestyl in August and November (Pl. I *d*₁). Consequently one should expect growth and differentiation of the deferent ducts in control birds killed in January. However, this is not the case (Pl. II *e*). Making use of Champy-preparations we found (Pl. II *c*) that the special interstitial cells (black in Pl. II *d*) are exclusively lipid cells; these are also visible (Pl. II *b*) in the testis-section of the same control bird, where a concentration of sudanophil elements in definite parts of the interstitial tissue is distinct.

Secretion cells, which in our opinion are the essential source of the male sex hormone, have not yet been formed, which explains the slight development of the deferent duct (Pl. II *e*).

The size and histological structure of the intertubular tissue and of the deferent ducts of *gestyl-treated* January birds differ little from those of *gestyl-treated* August and November birds. In the January birds the number of lipid cells is larger (Pl. II *b*₁) than in the others (Pl. I *b*₁); however, secretion cells are also present. Consequently the deferent ducts are abnormally well developed for the time of the year. The results of our experiments in August and November have therefore been confirmed by those carried out in January.

3. *In the Mature Stage*

In May chaffinches are completely mature sexually; an effect of gestyl-injection was not visible macroscopically: both experimental and control birds possessed testes of 6.5 mm. The histological differences were also small.

(a) *Testis-tubules.* Pl. III *d*, drawn from a testis-section of a sexually mature chaffinch, shows that the enlargement of the testes in the reproductive season is only the result of the enormous development of the testis-tubules. (When comparing one must take into account that in Pl. III *d* the radius of the semicircle is proportionate to *half* the average lengths of the axes of the testis.)

The histological structure of the testis-tubules is the same in experimental and in control birds: in both cases spermatogenesis is complete with many ripe sperms, their heads arranged in bundles on Sertoli cells (Pl. III *c*).

Consequently gestyl-injection in chaffinches in May does not have any influence on spermatogenesis.

(b) *Intertubular tissue and deferent ducts.* Contrary to the opinion of most of the earlier authors (e.g. Watson, 1919), who thought that the quantity of intertubular tissue in the ripe testis is much smaller than in the quiescent one, Benoit (1923*b*) holds the view—with which we fully agree—that this only seems to be so. Though the intertubular spaces have become very narrow, the total volume of the intertubular tissue need not necessarily have decreased. Moreover, Benoit points to the fact that the quantity of the special interstitial cells, not the size of the intertubular tissue, is of primary importance for the endocrine function of the testis.

From Pl. III *c* it follows that the intertubular tissue of the mature May-testis is histologically different from that of other seasons. Besides differentiated connective tissue cells and blood-vessels many lymph-spaces occur, lacking in the intertubular tissue of testes of other seasons. We cannot give any explanation of the physiological significance of these lymph-spaces; we will only state that they are also common in rodents (Sluiter, 1945). In addition, special interstitial cells are present in the intertubular tissue of the ripe testis (Pl. III *d*, black), their total number being about the same as after gestyl-administration in May. Most of them are lipoid cells which are still more numerous in the experimental (Pl. III *b*₁) than in the control birds (Pl. III *b*). Moreover, some secretory cells are present (Pl. III *c*). From this observation it is obvious that a small number of secretion cells suffices to produce the small quantity of sex-hormone needed to maintain the activated stage of the male genital ducts (Pl. III *e*).

Our observations clearly show that the effect of gestyl-injection on the testis in May is very slight or lacking; this is not surprising, as the mature testis-stage is normally also reached at this time of the year.

DISCUSSION

1. *Hormonal Regulation of Spermatogenesis*

In 1931 Schockaert and in 1935 Witschi and Keck demonstrated that in birds, spermatogenesis is normally caused by gonadotrophins of hypophyseal origin. With preparations derived from mammalian hypophyses complete spermatogenesis was induced in juvenile domestic ducks and in adult sparrows in the quiescent stage respectively.

As to investigations in which gonadotrophins, prepared from pregnant urine, were administered, the results do not agree. Schockaert (1933) got no results in cockerels and juvenile ducks when using such a preparation; Lahr, Riddle, and Bates (1941), however, observed a definite influence on the testes of pigeons and doves. Moreover, de Fremery (1941) induced complete spermatogenesis in sparrows in February, with the aid of pregnyl, a preparation from pregnant urine. Neither did investigations with the third type of gonadotrophins, i.e. those derived from pregnant mare serum, give corresponding results. The present authors have recently communicated that gestyl, a preparation from pregnant mare serum, has no influence on the

spermatogenous tubules of cockerels (Sluiter and van Oordt, 1947). Witschi and Keck (1935) had the same negative result after daily administration of 50 R.U. of another chorionic gonadotrophin in male sparrows, but de Fremery was able to induce 'a similar development of the testicles' (1941, p. 188) in sparrows with gestyl and pregnyl, i.e. he obtained complete spermatogenesis. This observation was confirmed by Pfeiffer and Kirschbaum (1943), also in sparrows, and by Lahr, Riddle, and Bates (1941) in pigeons and doves.

The present investigation shows distinct histological changes in the testis-tubules of chaffinches after gestyl administration; spermatogenesis, however, is not induced. Consequently our results do not harmonize with those of de Fremery, who used the same preparation and a related species. This difference in result may partially be explained by the quantity of hormone administered: de Fremery's sparrows got each 1,000 I.U. of gestyl in total, whereas our chaffinches received only half this amount. But this does not explain the fact that in the testes of our experimental birds no initial stages of spermatogenesis were induced; in our birds the diameter of the spermatogenous tubules only increased in size, due partly to the formation of a lumen, and partly to the plasma of the cells becoming distinctly inflated. The same histological structure is never found during normal spermatogenesis and therefore cannot be considered as an initial stage of spermatogenesis.

This observation clearly shows that after hormone injections neither the sizes nor the weights of the testes are indications of an active spermatogenetic stage.

2. *Function of the Intertubular Tissue of the Testis*

In the literature dealing with the interstitial cells—often called 'true Leydig cells'—of the testes of normal birds contradictory opinions about their function are met with. In some cases even their presence in one bird-species is denied by one author, whereas it is stated by others. For instance, Benoit (1936) found interstitial cells in the normal duck, in which they were not traced by Schockaert (1931), and in the sparrow they were described by Pellegrini (1925) but not observed by Pfeiffer and Kirschbaum (1943).

For these contradictions two causes may be put forward:

- (a) There is no agreement on the definition of the interstitial cells.
- (b) To demonstrate presence and function of the interstitial cells the ordinary laboratory routine techniques are insufficient.

As most of the investigators of mammalian or avian testes were not aware of the second cause or did not agree with it (Pfeiffer and Kirschbaum, 1943, p. 219), we think it superfluous to compare their results with ours.

The normal cytology of the interstitial cells of the testes of an exotic bird-species with a distinct sexual cycle, i.e. in the 'combassou' (*Vidua* sp.) has been described by Benoit (1923a). His technique, the only one suitable to

study these cells, was the same as ours: fixation in an osmic liquid and staining according to Altmann's technique. He stated that in the beginning of the progressive stage of the testis a number of cells in the intertubular tissue assume a glandular appearance, which process accentuates as the spermatogenesis makes progress. When the testis-tubules have attained their maximum size, cells with glandular structure, secreting the male sex hormone, are still present in the intertubular spaces, according to Benoit.

Only in one point do Benoit's and our conclusions differ essentially: Benoit does not make a sharp distinction between lipoid cells (i.e. cells of Leydig) and secretion cells, which he takes together as 'tissue glandulaire', whereas the present authors have demonstrated (Sluiter and van Oordt, 1947) that in the testis of the domestic fowl the sex hormone is only produced by the secretion cells. Our results in chaffinches, described above, are in full agreement herewith (cf. p. 6).

Another question is, whether the lipoid cells can pass over into secretion cells, as Benoit (1929) thinks. According to us the following points are in favour of this opinion:

1. Notwithstanding the fact that in all birds so far investigated the lipoid cells possess a very high content of lipoids, the structure of their nucleus, their size, and their large quantity of fuchsinophil elements are very similar to those of secretion cells.
2. The lipoid, which is present in these cells in avian and mammalian testes, consists, at least partly, of cholesterol; in the present investigation this also was found by us by the aid of Schultz's cholesterol test. As cholesterol is the substance from which the synthetic androgens are prepared, it is not unthinkable that the lipoid cells are destined to pass over into hormone-secreting cells. However, in our opinion this is by no means proved.

Against it the following facts can be raised:

1. In Stieve's fattening experiments (1923, 1926) the quantity of lipoid cells in the testes of geese and cocks was increased considerably, but an increased hormone-production could not be established.
2. It was found by many authors that the testes of the animals investigated did not contain 'classical Leydig cells' (i.e. our lipoid cells), whereas male sex-hormones were formed in these organs.

From the above we may conclude that, as yet, lipoid and secretion cells must be distinguished from each other as two cell types with different functions. As to the lipoid cells, it is certain that their presence in the intertubular testis-tissue is a reflection of the trophic role of this tissue, supporting either the endocrine or the generative or both testis-functions. In chaffinches the trophic activity of the intertubular tissue is obviously subject to the seasonal periodicity.

ACKNOWLEDGEMENTS

Our thanks are due to the Directors of Organon N.V., Oss (Holland), who kindly furnished us with gestyl.

The Royal Society kindly made a grant towards the cost of publication of the Plates.

SUMMARY

1. Male chaffinches (*Fringilla coelebs*) were treated with gestyl, a gonadotrophin prepared from pregnant mare serum, in different seasons; using different techniques their testes and deferent ducts were histologically studied after autopsy on 11 August, 30 November, 28 January, and 5 May.

2. After Champy-fixation and Altmann-staining two types of interstitial cells can be distinguished in the intertubular tissue of sexually active chaffinches: lipoid cells (= Leydig cells) and secretion cells.

3. Results of gestyl-administration: In summer- and winter-birds (Pl. I) whose testes are in the resting stage, the testis-tubule diameter shows a strong enlargement, which is partly due to the plasma of the cells being distinctly inflated; spermatogenesis does not take place. In the intertubular tissue lipoid and secretion cells appear in abnormally large numbers. In 10 days the deferent ducts pass over from the quiescent into the fully activated stage. In spring-birds (Pl. III), being in the reproductive stage, the administration of gestyl has practically no effect. In this stage the intertubular tissue also contains both lipoid and secretion cells.

4. From the results mentioned under 3, and the fact that in the control bird of 28 January, being in the beginning of the progressive stage (Pl. II), many lipoid cells were found, whereas its deferent ducts were still quiescent, it is concluded that only the secretion cells produce the male sex-hormone. The lipoid cells, which amongst others contain cholesterol, possess only a trophic function.

5. The difference in reaction of the seminiferous tubules of birds to chorionic and hypophyseal gonadotrophins as well as the function of the interstitial cells are discussed. Most opinions on the last-mentioned subject are not sufficiently well founded, as the investigators used routine techniques only for the cytological investigation of the interstitial tissue.

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DESCRIPTION OF PLATES

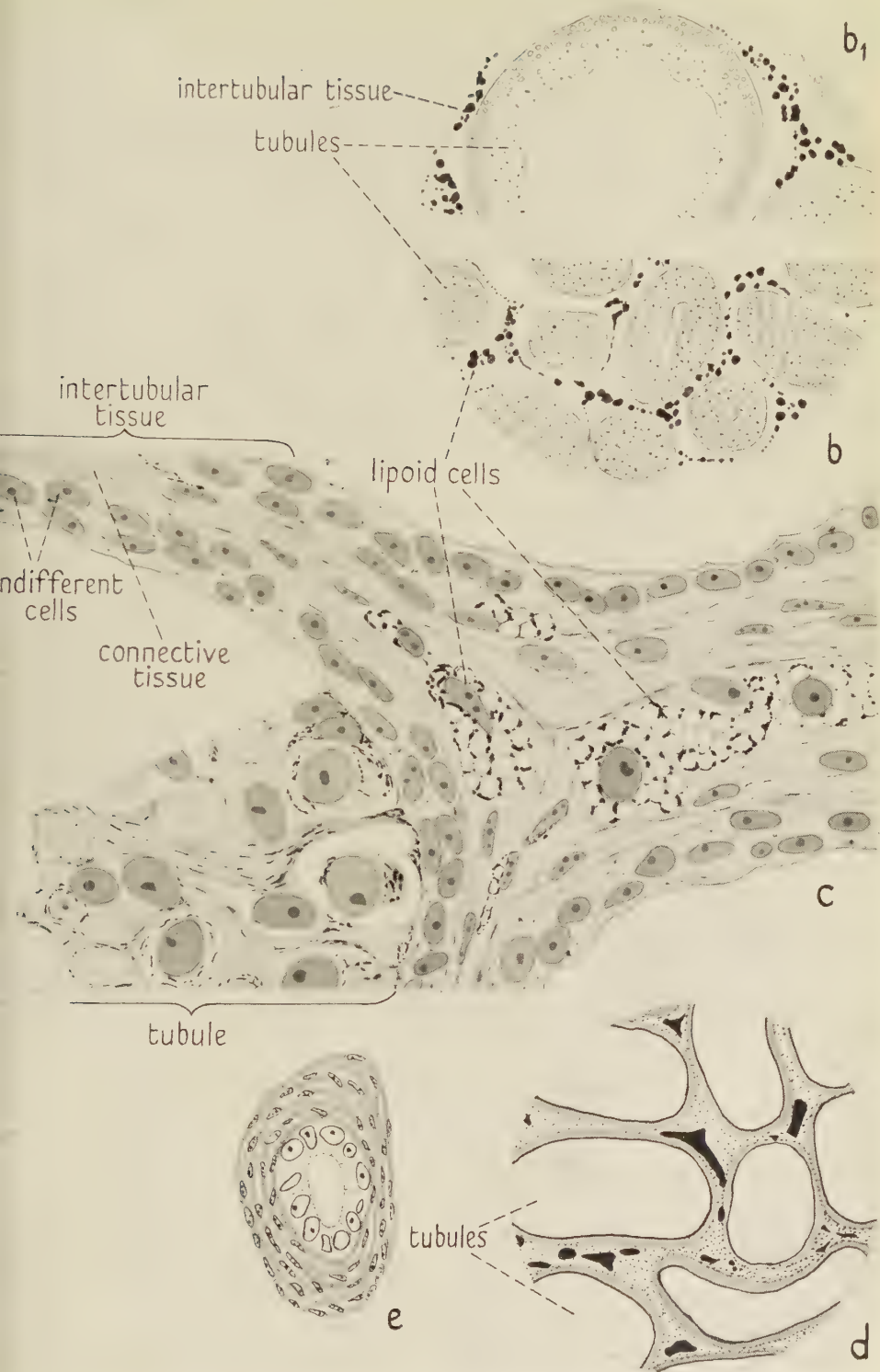
PLATE I. Testis- and deferent duct-sections of chaffinches, killed in August and at the end of November. *a* and *a*₁, sections of testis-tubules stained with haemalum-eosin, respectively before and after gestyl-treatment ($\times 500$); *C* and *C*₁, testis-sections after Sudan III-staining, respectively before and after gestyl-treatment ($\times 100$); *c* and *c*₁, the same after Champy-fixation and Altmann-staining, respectively before and after gestyl-treatment ($\times 1,000$); *d* and *d*₁, the same (schematic, $\times 100$; testis-tubules white, indifferent interstitial cells grey, connective tissue dotted, differentiated interstitial cells black); *e* and *e*₁, sections of deferent ducts, stained with haemalum-eosin before and after gestyl-treatment ($\times 500$).

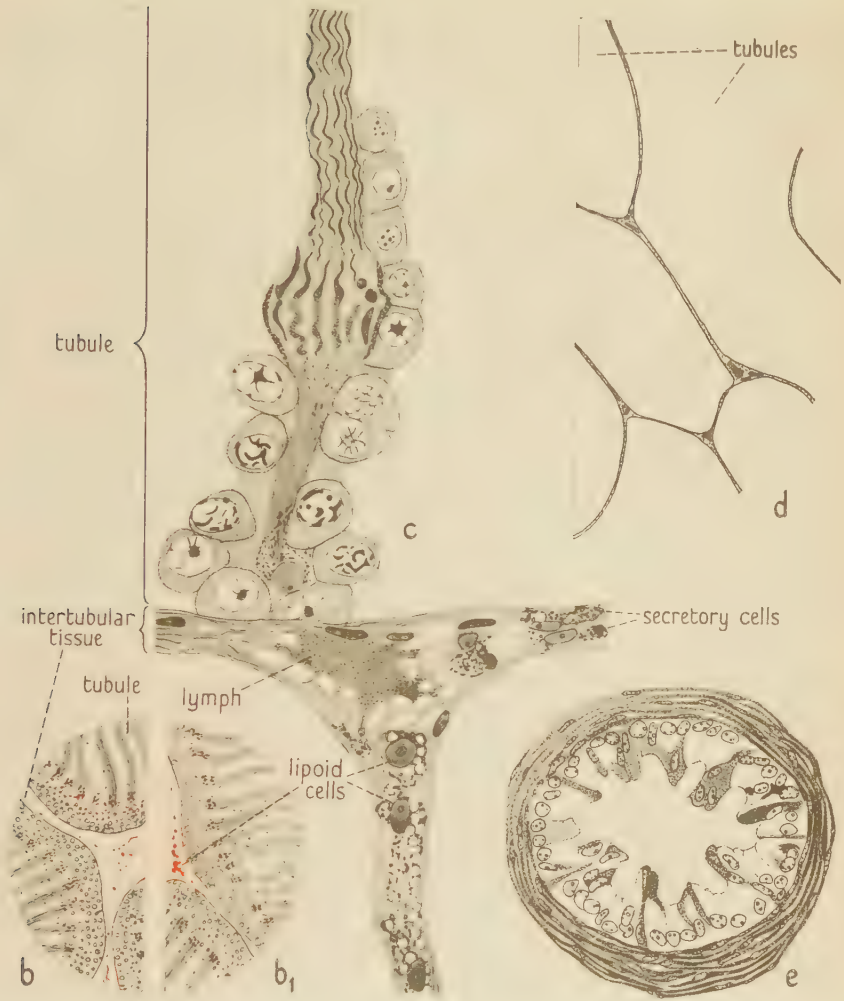
PLATE II. Testis- and deferent duct-sections of chaffinches killed at the end of January. *b* and *b*₁, testis-sections after Sudan III-staining, respectively before and after gestyl-treatment ($\times 200$); *c*, section of control-testis after Champy-fixation and Altmann-staining ($\times 2,000$); *d*, the same (schematic, $\times 200$; for white, grey, dotted, and black areas cf. Pl. I *d* and *d*₁); *e*, section of deferent duct of the same control bird after haemalum-eosin staining ($\times 1,000$).

PLATE III. Testis- and deferent duct-sections of chaffinches killed at the beginning of May. *b* and *b*₁, testis-sections after Sudan III-staining, respectively before and after gestyl-treatment ($\times 100$); *c*, section of gestyl-treated testis after Champy-fixation and Altmann-staining ($\times 1,000$); *d*, the same (schematic, $\times 50$; for white, dotted, and black areas cf. Pl. I *d* and *d*₁); *e*, section of deferent duct of a control bird, stained with haemalum-eosin ($\times 500$).



J. W. SLUITER AND G. J. VAN OORDT—PLATE I





J. W. SLUITER AND G. J. VAN OORDT—PLATE III

The Structure of Whale Blubber, and a Discussion of its Thermal Properties

BY

D. A. PARRY

(From the Department of Zoology, University of Cambridge)

With one Plate

AS a warm-blooded animal, a whale must be adapted not only to prevent an excessive loss of heat but also to control the rate of heat loss in relation to changes in its metabolic activity and in the temperature of its surroundings. This paper contains an account of whale blubber considered from this point of view. The first part is devoted to the morphology of blubber, particular attention being paid to the blood circulation which does not appear to have been previously described; and the second part contains a discussion of the efficacy of blubber as a thermal insulator of controllable conductance. The results are based on a study of the Common Porpoise (*Phocaena phocaena*), and the Blue and Fin Whales (*Balaenoptera musculus* and *B. physalus*).

The term 'blubber' is in popular and commercial use to denote the superficial tissues of whales and seals, which form a compact layer loosely fastened to the underlying muscle and easily stripped off for commercial purposes. Blubber comprises the animal's epidermis, dermis, and hypodermal tissues; and in the following account the word will always be used in this sense. The term 'integument' is used to refer to these tissues in other mammals.

GROSS MORPHOLOGY

Area

The surface area of whales and porpoises was found by measuring the circumference or semi-circumference at intervals down the body and finding the area contained by the smooth curve drawn through these values plotted against length. Flukes, dorsal fin, and flippers (measured for the porpoise only) were traced on squared paper.

Phocaena phocaena. One male, 155 cm. long, was measured, the circumference being taken at intervals of 15 cm. The results were:

Area of body only . . .	9,950 cm. ²
„ flukes . . .	820 „
„ fin . . .	250 „
„ flippers . . .	540 „
Total area . . .	11,560 „

Assuming that the surface area is proportional to the square of the length, that is to say, $A = Kl^2$, we have:

$$K = 0.45 \text{ for the total area,}$$

$$K_1 = 0.39 \text{ for the body area alone.}$$

The value for K is in good agreement with that calculated from Gray's data (Gray, 1936) which gave $K = 0.44$.

Balaenoptera physalus. Measurements were made of one adult whale and several foetuses, the results being as follows. The figures refer to the body area alone, excluding fin, flukes, and flippers.

Length	Measurement interval	Area	K_1
19.8 m.	3 m.	137 m. ²	0.35
308 cm.	25 cm.	32,330 cm. ²	0.34
245 cm.	„	20,280 cm. ²	0.34
240 cm.	„	20,710 cm. ²	0.36

These figures give a mean value for K_1 of 0.35. This compares favourably with the value of $K_1 = 0.37$ derived from Laurie's data (1933) which were based on the assumption that a whale can be regarded as two cones fastened together at their bases.

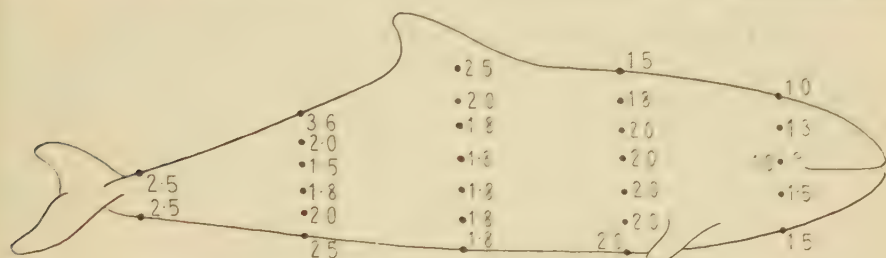
Thickness

Phocaena phocaena. The blubber thickness of a 155-cm. male was found both by direct measurement and indirectly from the area, weight, and density. The results of direct measurement are shown in Text-fig. 1 and give a mean thickness of 1.8 cm. The same figure was obtained by the other method, the density having been determined as 0.98 gr./c.c.

Balaenoptera physalus. It is difficult to study the distribution of blubber thickness under the conditions on board a factory ship, but from personal observations and those kindly made by Dr. M. Begg (Biologist, Factory Ship *Balaena*, 1946-7) I found that the thickness on the flank, level with the dorsal fin (the standard measuring-point of the Discovery Investigations), was approximately the mean value for the whole surface. It is now possible to amplify this conclusion as I have the advantage of a personal communication from Dr. E. J. Slijper embodying the results of extensive measurements made during 1946-7 and 1947-8. From these measurements I have calculated the mean thickness in two ways. Firstly, the outline of a whale was drawn on graph-paper so that it was divided into about eighty squares. Blubber thicknesses were marked in or interpolated, and the mean found from the sum of the product of areas and thicknesses. This gave a mean of 1.1 times the thickness at the standard place. The method may be suspected of error owing to the foreshortening of the dorsal and ventral surfaces, but as both the thickest and thinnest blubber is to be found on these surfaces (dorsal and ventral surfaces of the tail, and ventral surface of the throat and chest, respectively), the

resultant error is unlikely to be great. This contention is supported by the fact that the second method of calculation gave the same result as the first. This method was to find the mean of an equal number of thicknesses measured or interpolated along the dorsal, lateral, and ventral lines, and effectively allots equal areas to the dorsal, ventral, and lateral surfaces.

Extensive data are available concerning blubber thickness at the standard place. It varies with locality, time of year, and size of whale. Mackintosh and



TEXT-FIG. 1. *Phocaena phocaena*. Depth of blubber in centimetres.
Length of animal: 155 cm.

Wheeler (1929) give measurements made at South Georgia, while the following are taken from Dr. Slijper's communication and come from whales caught on the Antarctic pelagic whaling grounds:

	Dec.	Jan.	Feb.	March
	cm.	cm.	cm.	cm.
Blue whales, ♂ 74'-80' .	11.7	10.6	12.6	12.1
" " ♀ over 80' .	15.2	14.0	16.0	16.4
Fin whales, ♀ 65'-70' .	7.8	9.6	11.2	12.7

MICRO-ANATOMY AND HISTOLOGY

Phocaena phocaena

Material

The following account is based on the blubber of a 155-cm. male porpoise which after death had spent 2-3 days in transit in Great Britain at air temperature (October) and a further 4 days in a cold room (0° C.) while a plaster cast was made, during which process local warming of the blubber must have occurred. Despite this treatment a considerable amount of cytological detail was preserved, as will be seen from the photomicrographs. The animal fortunately died with its peripheral blood-vessels dilated, and the blood remaining in the vessels enabled their course to be made out.

Methods

After the post-mortem delay described above, small pieces of material were fixed in Susa or in 5 per cent. formalin, both fixatives giving good results. For preparations of the epidermis, paraffin embedding was satisfactory and sections down to 4 μ were obtained; but this method was unsuitable for the

dermis and hypodermis as it hardened the connective tissue and dissolved away the fat so that the tissues were badly torn and distorted during cutting. Lendrum's method of softening with phenol (Carleton and Leach, 1938, p. 39) was tried unsuccessfully, and frozen sections were finally resorted to. Good preparations down to 20μ were obtained, and little difficulty was experienced in making up complete series by transferring each section to a slide treated with gelatin, and exposing the full slide to formalin vapour (Pantin, 1946, p. 26).

The epidermis was well stained by Heidenhain's haematoxylin. More difficulty was experienced with the other tissues, where a stain was required which would not obscure the red blood in the vessels and would show up the vessel walls so that arteries could be distinguished from veins. Mallory's triple stain, and Masson's trichrome stain, gave unsatisfactory results, and the required contrast between blood and tissues was finally obtained by staining lightly with 1 per cent. water-soluble aniline blue.

Tissue Components

The blubber of the porpoise is composed (Pl. I, fig. 1), like the integument of other mammals, of epidermis, dermis, and hypodermal tissue. Its most distinctive features are the thickness and almost exclusively fatty nature of the hypodermis, and the absence of hair, skin glands, and sense organs. The following account deals separately with the three tissue layers, and then considers the blood-supply. No sensory or vasomotor nerves have been found.

Epidermis

(i) *Micro-anatomy.* The thickness of this, the most superficial layer, varies little over the body, being 3.25 mm. or $\times(2 \times 10^{-3})$ of the body length. It varies in colour from white to dark grey, being responsible for the colour of the animal. The deeper half of the epidermis is penetrated by upward extensions of the dermis in the form of 'dermal ridges' running parallel to the long axis of the body, from the summits of which arise the dermal papillae. The section illustrated in Pl. I (fig. 1) is cut at right angles to the long axis of the body so that the ridges are cut transversely; the second ridge from the right-hand side has been cut between two papillae while all the others have papillae rising from them so that the distinction between the two is not evident. Pl. I, fig. 3 shows a transverse section through the ridges, and fig. 2, cut more superficially, shows the papillae which have arisen from the ridges. This arrangement differs from the condition found in most mammals where the papillae arise directly from the base of the epidermis.

There are approximately 25 papillae per sq. mm., which is about half the mean density found in man (Lewis, 1927, p. 253).

(ii) *Histology.* The epidermis is composed of two layers, the superficial *stratum corneum* (unstained in Pl. I, fig. 1); and a deeper layer which on comparative grounds will be called the *stratum germinativum*, although no mitoses have been observed. The transition between the two layers is characterized by a

flattening of the cells parallel to the surface, a thickening of the cell walls or inter-cellular substance, and a reduction of nuclei and cytoplasm. There is no *stratum granulosum* or *stratum lucidum*, a condition not unusual in other mammals.

As in other mammals the *stratum germinativum* may be subdivided into 'cylindrical cells' with large nuclei, lining the dermal ridges and papillae; and polygonal 'prickle cells' with well-developed intercellular and intra-cellular fibrils, lying superficial to the papillae.

In the pigmented parts of the blubber, pigment granules occur in the epidermis; most densely in the cylindrical cells of the *stratum germinativum*, especially those immediately bordering the ridges and papillae; and less densely in the prickle cells. Pl. I, figs. 2-4 show the granules in unstained sections.

Dermis

This consists of a mat of connective tissue fibres parallel to the surface, very dense at the base of the epidermis (Pl. I, fig. 1), and becoming less dense and invaded by fat cells at deeper levels where it merges into the underlying hypodermis. Its thickness is about 0.34 mm., or $\times (0.22 \times 10^{-3})$ of the body length. This tissue is formed predominantly of white fibres, staining characteristically with v. Giesson's stain. No elements took up orcein, but a few elastic fibres were recognized in some preparations.

Hypodermis (note: only a fraction of the total thickness of the tissue is shown in Pl. I, fig. 1)

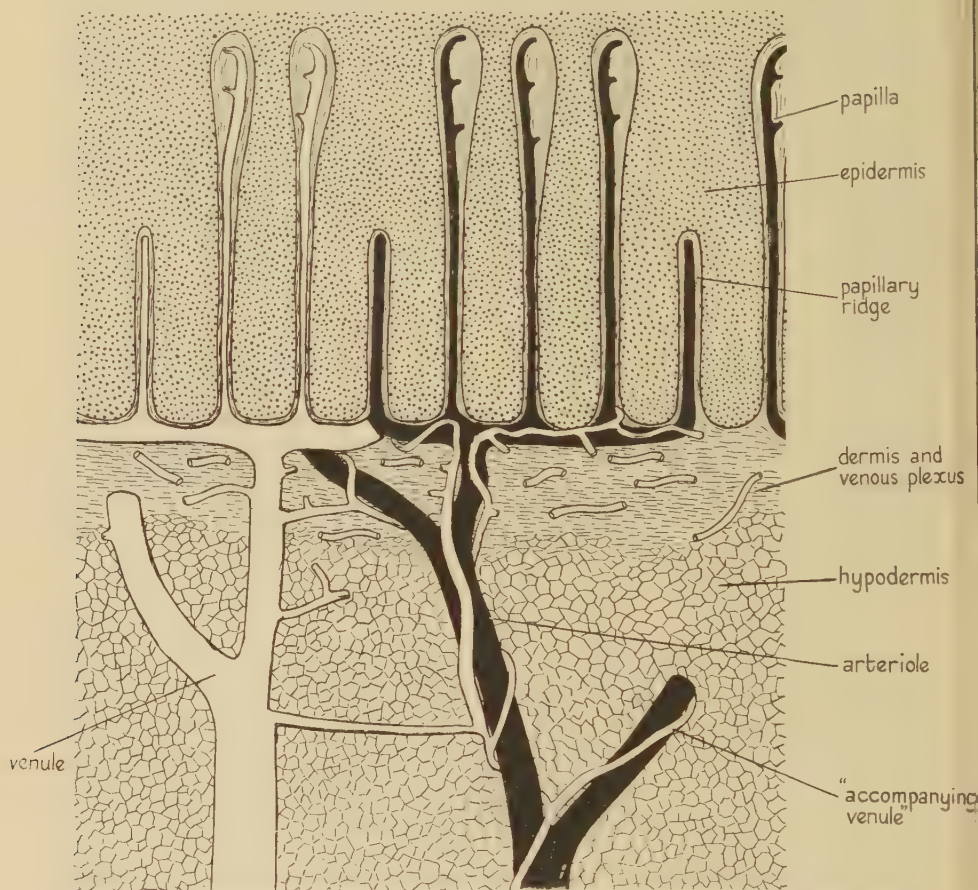
This tissue is composed almost entirely of fat cells, with occasional bundles of white fibres running irregularly among them (Pl. I, fig. 1). The great development of this layer is responsible for the thickness of blubber as a whole, and for its commercial value. It corresponds to the panniculus adiposus of that other almost hairless mammal, man; and presumably also to the thick layer of fat beneath the skin of the domestic pig.

Blood-supply (see Text-fig. 2, which has been constructed from a large number of serial drawings)

(i) *Arteries*. Arterioles run up through the hypodermis, branching as they do so; and through the dermis to the base of the epidermis. Here they run somewhat obliquely across the bases of the dermal ridges, giving off twigs which run up the ridges to join the capillaries in the papillae. The most careful examination has failed to show the arterioles giving rise to capillaries or forming a plexus in the hypodermis or dermis, neither are there any signs of arteriovenous anastomoses. The only course open to the blood appears to be up the twigs in the ridges to the capillaries in the papillae. Text-fig. 3 (a) shows an arteriole running through the dermis to the base of the epidermis. Pl. I, fig. 3 shows both arterial and venous twigs in the dermal ridges, running upwards, and also horizontally to supply the papillae arising from the

summits of the ridges. It is probable that even at this level, arterial and venous vessels are distinguished by the thickness of their walls.

(ii) *Capillaries*. Capillaries occur in the papillae (Pl. I, fig. 2) where the terminal twigs are probably joined together by several short capillaries, as



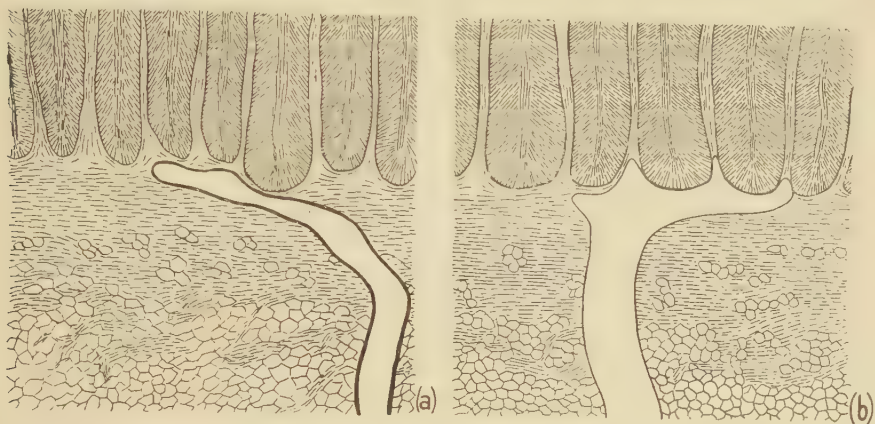
TEXT-FIG. 2. *Phocaena phocaena*. Blood circulation in the superficial regions of the blubber. To avoid confusion, arterial and venous vessels are not both shown in the same dermal ridge and papilla. Based on numerous serial drawings.

photomicrographs of living tissue have shown to be the case in man (see Lewis, 1927, p. 13). The diameter of the capillaries is about 15μ .

(iii) *Veins*. Venous twigs running down the dermal ridges are collected into venules which, like the corresponding arterioles, run obliquely across the base of the epidermis and then down through the dermis and hypodermis, joined by venules from adjacent parts of the skin. Text-fig. 3 (b) shows a vertical section through the dermis and base of the epidermis, with one of these venules running horizontally just below the epidermis collecting twigs from the ridges, and then turning to run down through the dermis. Pl. I, fig. 4, is a thick,

horizontal section through the extreme base of the epidermis, showing a collecting venule running just below it.

Unlike the corresponding arterioles, these venules are associated with an extensive plexus in the dermis and the transitional region between dermis and hypodermis. This plexus is shown in Pl. I, fig. 5. It appears to be a single network, with elements running in both the horizontal and vertical planes (see Pl. I, fig. 1), and so the short lengths of plexus shown in Text-fig. 2 must be imagined as inter-connected in planes above and below that of the drawing. This text-figure illustrates the fact that the venules preserve their identity



TEXT-FIG. 3. Vertical sections through the dermis and base of the epidermis showing (a) an arteriole, and (b) a venule, running up to supply the dermal ridges and papillae. Based on 100μ frozen sections, stained with aniline blue.

up to the base of the epidermis, connecting with the plexus by means of short branches; they do not divide up into the plexus which in turn gives rise to more superficial vessels. Thus blood from the papillae may run directly down through the dermis and hypodermis in the large venules; or it may flow through the narrow plexus vessels under the influence of small pressure differences between one part of the plexus and another. The diameter of the plexus vessels is about 30μ .

In addition to the large venules already described, which pursue an independent course through the blubber, there are much smaller venules, often not more than one, accompanying the arterioles in at least the superficial layers of the hypodermis, and in the dermis up to the base of the epidermis but not entering the dermal ridges (see Text-fig. 2). These vessels, like the large ones, contribute to the venous plexus, and are occasionally connected with the large ones by branches in the hypodermis.

Balaenoptera

The sample of the blubber of *Balaenoptera physalus* brought to England in a hard-frozen state for heat-conductivity measurements (see below) also

afforded material for histological examination. The peripheral blood-system could not be followed as it did not contain blood, but the micro-anatomy of the three tissue layers—epidermis, dermis, and hypodermis—was similar to that of *Phocaena* with the exception of a peculiar vacuolation in the epidermis which we suspect to be an artifact due to freezing (see next paragraph).

We have also examined a sample of the blubber of *Balaenoptera musculus* kindly supplied by Dr. Michael Begg, who fixed it in formalin shortly after the capture of the whale. The micro-anatomy of the tissue layers was also similar to that of *Phocaena*, and the vacuolation noted above did not occur. The blood-system has not been traced out in detail, but the main elements found in *Phocaena* were all present: arterioles running through hypodermis and dermis to give off twigs to the dermal ridges; collecting venules receiving twigs from the ridges and running down through dermis and hypodermis; and plexus in the dermis.

DISCUSSION OF THE MICRO-ANATOMY AND HISTOLOGY

A few previous accounts exist of the blubber tissues, excluding the vascular system. That of Bonin and Vladykov (1940) deals with the Beluga (*Delphinapterus leucas*) where the dermis is relatively better developed than in our species, so as to be of commercial importance in the manufacture of shoelaces. These authors do not mention the dermal ridges; otherwise their account is in good agreement with ours. Stiglbauer (1913: *Delphinus delphis*) also does not describe the dermal ridges, nor does he find 'prickle cells' in the epidermis. Japha (1907: *Balaenoptera* spp.) gives a clear account of the ridges, from which Schumacher (in Bolk *et al.*, 1931) has made a good perspective drawing (Fig. 248, p. 468). In other respects, too, we are in general agreement with Japha, and his account of five different layers in the epidermis, as compared with our three, seems to be a refinement of description rather than a difference of substance.

We know of no other account of the blubber vascular system. This differs from that in the integument of man (Spanholtz, in Lewis, 1927) where the plexuses are much more complicated: the arterioles break up into two separate ones at different levels, while the venules divide up into no less than four. The 'accompanying venules', which we have described as running beside the arterioles, do not occur in man.

THE THERMAL PROPERTIES AND FUNCTION OF BLUBBER

Blubber regarded as a Heat Transmitter

Blubber is generally thought of as a protection against cold, but the integument of a homothermous animal is much more than a mere passive insulator. It actively controls the outward passage of heat, and so preserves the deep body temperature despite changes both in the rate of production of heat by the animal, and in the thermal characteristics of the environment.

In whales, no less than in terrestrial animals, is such a control of heat loss necessary. Variation in the rate of swimming must be accompanied by a considerable variation in heat production; and whereas most whales probably undertake irregular wanderings which carry them over at least a moderate range of temperature, the rorquals undergo a twice-yearly migration between polar and tropical waters, involving a temperature change of no less than 25° C.

The difference in the environment of whales and terrestrial animals carries with it necessary differences in the methods of heat-regulation. The latter lose heat by three channels: evaporation, controlled by the sweat glands and breathing; and convection and radiation, controlled by the surface temperature which is in turn influenced by vaso-regulation. In addition, habitat selection plays an important role. A whale, on the other hand, cannot sweat or seek shelter, neither can it radiate to an environment which is within a degree or two of its own surface temperature. It can lose heat solely by 'forced' convection to the water flowing past it, and we have shown that blubber contains a blood-circulation by control of which this heat loss could be regulated. The system will now be considered in more detail.

Minimum Heat Transmission. It is known that in man exposed to cold the blood-flow through the skin is reduced almost to zero by constriction of the arterioles (Lewis, 1927; Forster *et al.*, 1946). It is not unreasonable to assume that this is also true of whales where similar arterioles exist, though it may be added that if a slight flow is always necessary for purposes of nutrition, the heat loss from this might be reduced by a return stream of blood through the 'accompanying venules'. This would short-circuit some of the heat and prevent it from being lost at the surface.

When the blood-flow through the blubber is negligible, heat loss is entirely due to passive conduction through the tissues and its rate is given by the expression:

$$\text{Rate of heat transmission} = \frac{\text{Blubber conductivity}}{\text{Blubber thickness}} \times \left(\text{Body temperature} - \text{Sea temperature} \right).$$

The conductivity and internal temperature were found as follows:

(i) *Conductivity.* A sample of whale blubber (*Balaenoptera physalus*) about 1 ft. square was hard-frozen a few hours after the capture of the whale, and maintained in this condition for about five months during transport to, and storage in, this country. It was then kept at just over 0° C. for a week to thaw out and at air temperature (October) for 3½ hours during transport to the National Physical Laboratory, where it was immediately put in the heat-conduction apparatus. The Laboratory report was as follows:

The sample of whale blubber submitted for the determination of the thermal conductivity was supplied in the form of two slabs each measuring 12 inches by 12 inches by 1¼ inches approximately. For the purposes of the test, the slabs were placed one on each side of a hot plate surrounded by a guard ring, the whole being clamped between two cold plates maintained at a constant temperature. The

temperatures of the hot and cold surfaces were measured by means of thermocouples. The heat input to the hot plate was obtained by observations of the watts dissipated in its heating coil. The results given in the table below refer only to the particular sample tested.

Whale Blubber

(Approximate density 65 lb. per cub. ft.)

Cold face temperature: 0°C .

Hot ,, ,, : 35°C .

Thermal conductivity : 0.00050 gr.-cal. per sq. cm. per sec. for
1 cm. thickness and 1°C . diff. in
temp.

(ii) *Internal Temperature.* That of a Blue Whale (*Balaenoptera musculus*) was measured 60 minutes after death, the thermometer being placed in a deep cut in the epiaxial muscle, level with the flipper, immediately after the blubber had been removed from that part. Three readings all gave the same result: 35.5°C ., which is likely to be nearer the true value than Laurie's figure (1933) of 35.1°C ., based on the measurement of twenty whales at a land station.

Substituting these values for the conductivity and internal temperature in the above expression, we have:

$$\text{Heat transmission (cal./sq. cm./sec.)} = \frac{5 \times 10^{-4}}{d} (36 - t_c),$$

where d = thickness of blubber (cm.) and t_c = environmental temperature ($^{\circ}\text{C}$.).

Two examples may now be considered. For a porpoise in which $d = 1.8$ cm., living in a sea at $t_c = 10^{\circ}\text{C}$. (e.g. around the British coast):

$$\begin{aligned} H &= \frac{5 \times 10^{-4}}{1.8} (36 - 10) \\ &= 72 \times 10^{-4} \text{ gm.-cal./sq. cm./sec.} \\ &= 260 \text{ kilo-cal./sq. m./hr.} \end{aligned}$$

For a rorqual in which $d = 8$ cm., living in a sea at $t_c = 0^{\circ}\text{C}$., the heat loss is 81 kilo-cal./sq. m./hr. The significance of these figures will appear when it is recalled that the basal metabolism of a wide range of terrestrial animals (including horse, man, pig, dog, and hen) is almost constant and equal to 45 kilo-cal./sq. m./hr. —a figure which has come to be regarded as generally applicable though its physiological basis is unknown (see Krogh, 1941). If this figure also applies to the whale then we must conclude that neither the porpoise nor the rorqual, in the environments considered above, can afford to remain at rest: despite their blubber they would suffer a net loss of heat. It also follows that for the heat loss to be reduced to 45 kilo-cal./sq. m./hr. in any whale at rest (independent of size or species), a layer of blubber with an average thickness of 14 cm. would be required, a thickness which is

actually encountered in the largest rorquals, and in the right whales (Balæ-nidae), whose 'rightness' as a target for the harpoons of an earlier age was in part due to their sluggishness compared with the rorquals. We are, of course, aware that some authorities are unwilling to grant to the above figure for basal metabolism the dignity of a physiological constant. Benedict (1938) puts forward a relation making metabolism per unit area increase with length, quoting figures as low as 12 kilo-cals./sq. m./sec. for the dwarf mouse, and as high as 85 kilo-cal. sq. m. sec. for the elephant. His conclusion may be criticized on the ground that it is based on very few data from animals at the two extremes of size; but even if it is correct it quite fails to endow a small whale such as a porpoise with a basal metabolism nearly large enough to compensate for its heat loss in temperate seas.

Maximum Heat Transmission. This will be achieved when the blubber arterioles are fully dilated and the mass flow of blood to the surface is maximal. Then it is most likely that much more heat is lost from the blood than through the blubber, so the latter can be ignored in comparison. The interesting consequence is that maximum and minimum heat losses are virtually independent: the achievement of a low minimum by the development of a thick layer of blubber does not affect the maximum which will depend on the maximum mass flow of blood through the superficial vessels and their spacial arrangement. This arrangement may be contrasted with that found among the majority of terrestrial homotherms where the insulation (fur or feathers) overlies the vascular tissue, so that an increase in the thickness of the former must reduce the maximum as well as the minimum heat loss.

BLUBBER REGARDED AS AN ENERGY RESERVE

It is well known that the blubber of the southern rorquals shows a seasonal variation in thickness, being thinnest at the beginning of the southern summer when the whales are returning to high latitudes after wintering nearer the equator. This may be regarded as effecting a necessary increase in the minimum rate of heat loss corresponding to the warmer environment. But it has been suggested that the whales find little to eat in the tropics and subsist on the stored energy of their blubber. A simple calculation shows that this suggestion is quite plausible. According to Mackintosh and Wheeler (1929, p. 370) the reduction in thickness is 0.1 per cent. of the body length. Using the previously found expression $A = 0.39l^2$ for the surface area, and assuming the density of blubber is unity and its energy content 9 kilo-cal./gm., it follows that the energy liberated during 6 months is at the rate of ($l^3 10^{-6}$) watts. Now basal metabolism at 45 kilo-cal./m.²/hr. demands $22^2 \times 10^{-3}$ watts; so it follows that a 0.1 per cent. reduction in blubber thickness would just pay for the basal metabolism of a 20-m. whale, while it would afford larger animals extra energy for movement.

This conclusion is not intended to be taken quantitatively; but it does give qualitative support to the idea that the blubber of rorquals functions as a significant energy reserve.

While this work was being done I was successively Whaling Inspector and Discovery Biologist in the Factory Ship *Empire Venture* (1945-6), and the recipient of a Senior Research Grant from the Department of Scientific and Industrial Research (1946-7). I am most grateful for the facilities which these appointments placed at my disposal. I also wish to acknowledge the willing assistance of the Director and Staff of the Scottish Marine Biological Association in securing and injecting the porpoise; and the readiness of the National Physical Laboratory to accept for conductivity measurements a specimen possessing somewhat unwelcome characteristics.

SUMMARY

1. The gross morphology, micro-anatomy, and histology of the blubber of the porpoise (*Phocaena phocaena*) and the rorquals (*Balaenoptera* spp.) are described.

2. If the surface area is given by Kl^2 , l being the overall length, then K is 0.39 in *Phocaena* and 0.35 in *Balaenoptera*, excluding fins and flukes.

3. Blubber consists of the whale's epidermis, dermis, and hypodermis. The hypodermis is relatively very thick and almost exclusively fatty, and in the species studied merges into the dermis which is mostly composed of white fibres. It extends into the epidermis as 'dermal ridges', from which the papillae arise. In the epidermis can be recognized: *stratum germinativum*, divided into the deep cylindrical cells and the more superficial prickle cells; and *stratum corneum*.

4. The vascular system is composed of arterioles running up to the base of the epidermis, giving rise to twigs which run up the dermal ridges to supply the capillaries in the papillae; and venules collecting twigs from the ridges and running down through dermis and hypodermis, connecting in the dermis with a venous plexus. Small 'accompanying venules' run with the arterioles to the base of the epidermis.

5. The conductivity of blubber is 0.00050 gm.-cal./sq. cm./°C./cm. and the deep body temperature is about 36° C. Thus in temperate and polar waters most whales lose heat at a greater rate than the basal metabolic rate of land homotherms, even when the blood-flow through the blubber is negligible. It is suggested that whales need to keep swimming in order to keep warm.

6. The vascular system in the blubber provides a mechanism for regulating heat loss.

7. It is shown that the energy liberated by the reduction in blubber thickness suffered by rorquals in the southern hemisphere during the winter is sufficient to meet at least a significant part of their total needs.

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EXPLANATION OF PLATE I

(All the preparations are of *Phocaena phocaena*)

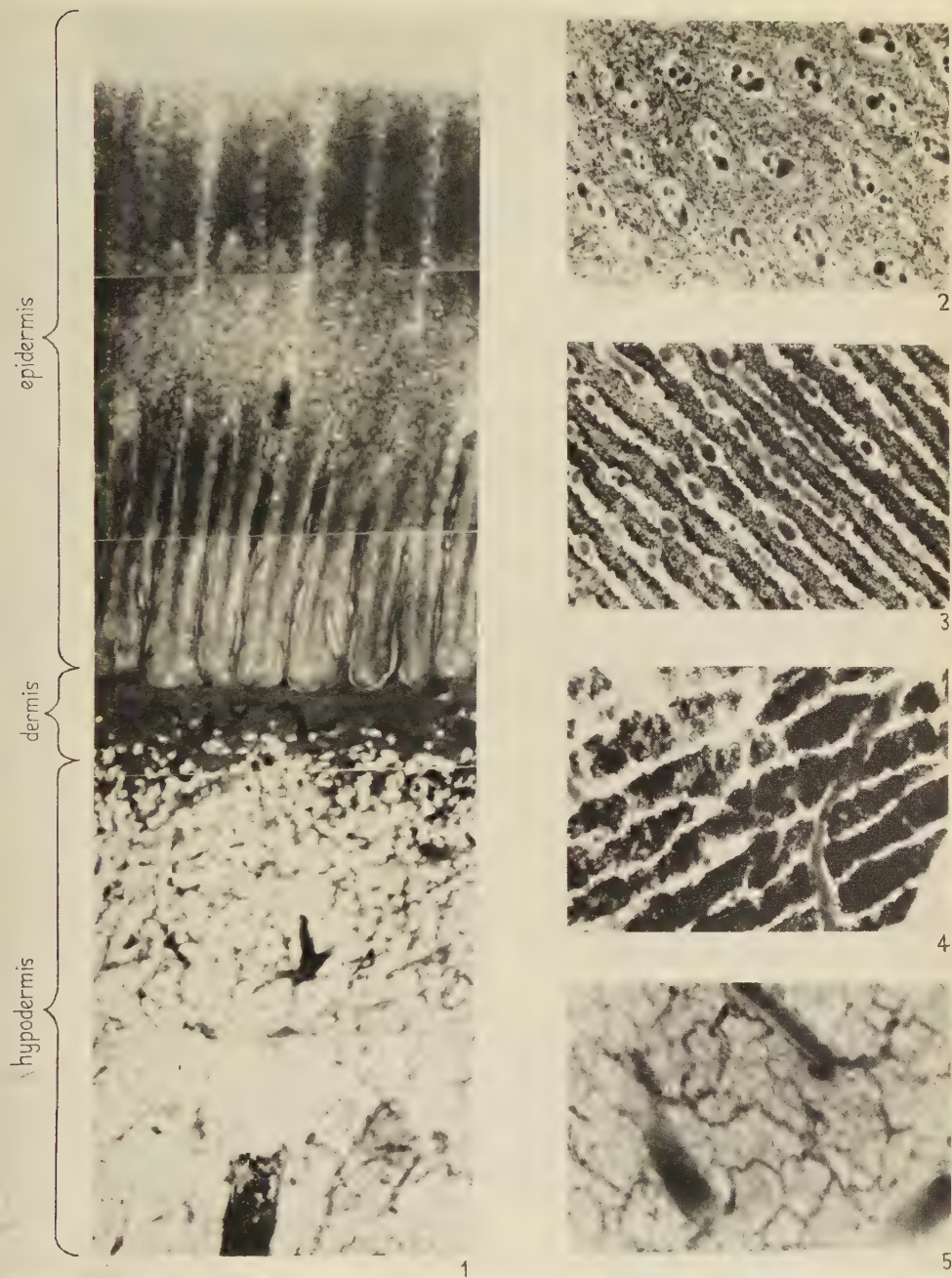
FIG. 1. Section through the epidermis, dermis, and superficial part of the hypodermis. Fixed in 5 per cent. formalin, several days *post mortem*. 100 μ frozen sections, stained with 1 per cent. aniline blue, W.S. ($\times 29$)

FIG. 2. Horizontal section through epidermis, showing dermal papillae with capillaries dark with blood. Fixed in 5 per cent. formalin, several days *post mortem*. 100 μ frozen sections, unstained. ($\times 29$)

FIG. 3. Horizontal section through epidermis, showing dermal ridges with venules dark with blood. Treatment as (2). ($\times 52$)

FIG. 4. Horizontal section through base of epidermis and dermal ridges, showing large venule. Fixed as (2); 200 μ frozen section, unstained. ($\times 52$)

FIG. 5. Horizontal section through dermis, showing venous plexus. Fixed as (2); 200 μ frozen section, unstained. ($\times 52$)



D. A. PARRY—PLATE I

Heliospora n.g. and *Rotundula* n.g., Gregarines of *Gammarus pulex*

BY

HELEN PIXELL GOODRICH

(From the Department of Zoology and Comparative Anatomy, Oxford)

With one Plate

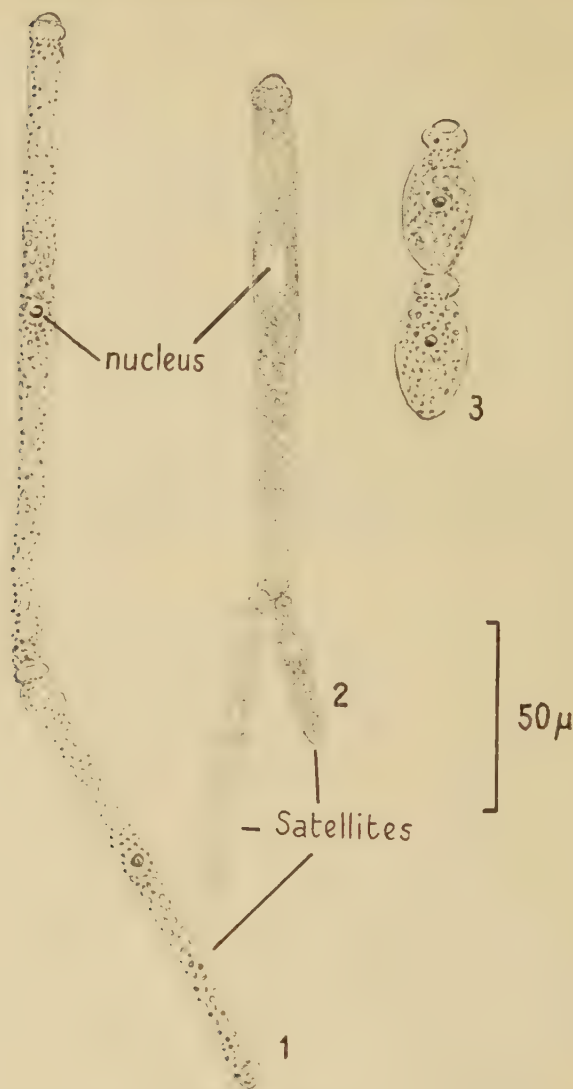
THE gregarines of *Gammarus pulex* L. have long been known, in fact they were some of the earliest of these Sporozoa to be observed (Text-figs. 1-3). However, so far, no adequate description has been given of the gametes and spores which are the most important stages from the systematic point of view.

Lady Muriel Percy, who worked in this Department from 1925 to 1929, helped in the investigation of spores by collecting infected *Gammarus* from different localities and isolating the Gregarine cysts for observations. From her notes and figures together with mine, which extend at intervals over the last quarter of a century, I have endeavoured to elucidate the life-histories of these two gregarines for which, I regret to say, it has been necessary to establish new genera as above.

Apparently Siebold was the first to study the trophozoites—some time in the early eighteen-forties. He did not describe them in his paper of 1839, as has been erroneously stated by several writers, but gave Kölliker permission to incorporate his description in a postscript to his (Kölliker's) paper of 1848. There, one can find quite recognizable figures (29 α and β) of a thread-like form, *G. longissima* and (γ) of a shorter, more rotund form. Siebold and others (Labbé, 1899) were uncertain whether the latter was a young stage of the former.

They were separated in the same year, 1848, by Frantzius, who attached the long thread-like gregarine of *Gammarus pulex* to the genus *Didymophyes* (just created by Stein for *D. gigantea* of *Oryctes* and *D. paradoxa* of *Geotrupes*), leaving the short form occurring with it as *G. gammari*?. He was followed by Diesing, 1859, who definitely stated that he excluded the latter from *G. longissima* and consequently this species *G. gammari* is now ascribed to Diesing owing to the uncertainty of Frantzius and the original observer Siebold. There is no doubt that '*longissima*' and '*gammari*' are their correct specific names. To find correct generic names has been more difficult. The thread-like form, even without knowledge of its cyst, could not be kept in the genus *Gregarina*, and Poisson (1921*b*), realizing that it also had no place in the genus *Didymophyes*, changed it to one *Uradiaphora* suggested by Mercier (1912*b* and *c*; see also p. 32 below) for a gregarine found in the fresh-water shrimp

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TEXT-FIG. 1. *Heliospora longissima* Sieb. A pair in syzygy.

TEXT-FIG. 2. A living primitive of the same with 2 satellites attached.

TEXT-FIG. 3. *Rotundula gammari* Diesing. A pair of associates.

Figs. 1 and 3 fixed Bouin's mixture and stained iron haematoxylin.

Atyaephyra at Nancy. Various characteristics of this genus, some contradictory, were given in Mercier's four papers of 1911 and 1912, e.g. 1912a p. 198: 'La syzygie à maturité sexuelle mesure de 5-700 μ de longueur. Kystes ovoïdes de 38-44 μ de long.' The latter and the spores appear more like those of *Rotundula* (see below, p. 33). The most surprising characteristic given by Mercier and said to be distinctive and from which he derived the

name, was the presence of a constriction towards the end of the deutomerite of the satellite forming 'une petite queue'. This the author suggested might be to prevent another trophozoite 'de venir troubler l'harmonie du couple'. This tail was said to be shaped like an appendix and to degenerate. Nothing of this sort has ever been seen by us in either of the gregarines of *G. pulex*. Sometimes the satellite itself of the long form is abnormally small and such a small one as well as one of more normal size are sometimes both attached to the primite (Text-fig. 2). We have evidence to show that both of these satellites may sometimes form gametes which undergo syngamy with those from the primite. Enough has, I think, been said, together with the characteristics to be given below, to show the necessity of making a new genus for this long-known thread-like gregarine of *G. pulex*, especially when the spores, considered the most diagnostic feature of gregarines, were found to be quite different from those of any known form.

These spores are provided with equatorial ray-like processes of the episporal coat and the name *Heliospora* therefore seems to be suitable.

Characteristics of *Heliospora* n.g.: Elongated, septate (polycystid) Gregarines having more or less spherical spores each provided with equatorial ray-like processes of the episporal coat.

Type species *Heliospora longissima* (Siebold) (see K  lliker, 1848) from gut of *G. pulex* L. Specific characteristics:

1. Elongated, filiform trophozoites, precociously associated.
2. No intracellular stage.
3. Small, button-shaped epimerite.
4. Epimerites and septa retained until the sporonts roll up for encystment.
5. Cyst approximately spherical, wall thin, transparent, and easily ruptured.
6. Gametes anisogamous, ♂ with delicate flagellum.
7. Spores slightly flattened at poles and at the equator the episore is produced into 6 long ray-like processes.

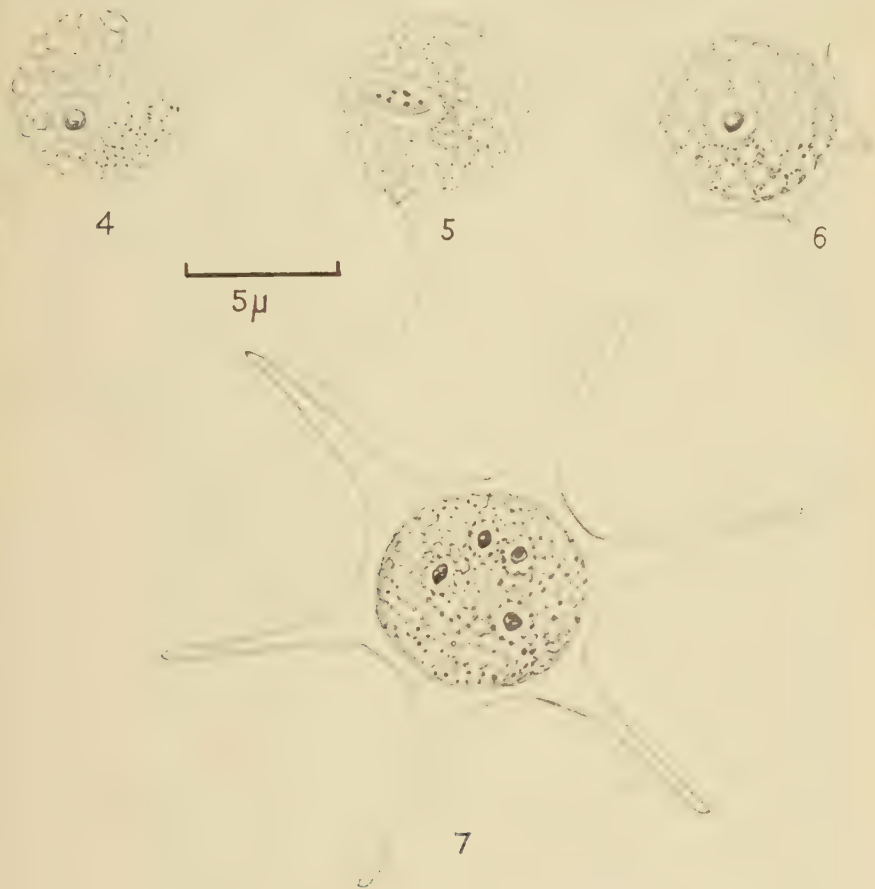
The thread-like trophozoites of *Heliospora longissima* may be up to 228μ long, but they naturally vary in length according to their state of contraction. They are sometimes only 8μ in diameter but may be double this width, especially in the region of the nucleus. The primite is often the longer but by no means always and our attempts to distinguish sex by intra-vitam stains have not given conclusive results. However, by watching development carefully, the male gametes have appeared to come from the smaller associates.

No intracellular stage has been found but young forms from 7.5 to 40μ or so long may be free in the lumen or cling to epithelial cells just as a satellite often presses its epimerite into the deutomerite of the primite to form a pair of different sex precociously. Many of these syzygies may be clustered together as so characteristic of gregarines. Towards the posterior end of the mid-gut, long pairs may be found folding themselves up with rather jerky movements,

finally forming cysts. These are $55-85\mu$ in diameter and through the thin wall the folds of the cuticle may be visible for some time. These cysts, often attached to a peritrophic membrane, pass out shortly before the host moults, or they may be left in the proctodaeum and cast off in the moult. With the cysts, trophozoites at various stages may be eliminated also but in our experience they do not develop into cysts outside. The loss of many parasites in this way at moulting seems to account for the poor infections often found and the habit shrimps have of eating their moults may also destroy some unripe cysts. Perhaps the best way of finding cysts is to place a single pair of *Gammarus* in a bowl of water shortly before young are due to be expelled from the ♀'s brood pouch; after this the ♂ helps her to moult and, *if infected*, cysts may be found adhering to the bowl. To catch the ♂ moulting is more difficult. When the cysts are evacuated in water, they sink and attach themselves to the bottom of the bowl by an adhesive secretion. By means of a needle or fine brush they can be lifted and mounted in a hanging drop, but on reaching the surface they, of course, tend to be detached and should be caught in a small pipette before again reaching the bottom of the bowl. With care these thin-walled cysts can be mounted unharmed and watched developing under high magnification through their transparent walls. In this way the accompanying time-table was worked out, see diagram (pl. I). There does not seem to be any definite time of day or night for moulting nor for the extrusion of cysts, but the one drawn (stage 1) was one of the first to be studied and we have been able to foretell the time of the dance on other occasions. It begins some 11 hours after the elimination of the cysts. It will be seen that the partition between the two associates (stages 1 and 2) soon disappeared but the cytoplasm again collected round each nucleus until division started some 3-4 hours later. During the next 3 hours these nuclei arranged themselves on the surface and there continued their division. About 3 hours later again some cytoplasm collected round each nucleus and the gametes became separated, and soon after the slight movement began. So far as we could make out only the smaller gametes (♂) had active movement, and this was due to a very fine flagellum which persisted in the zygote (Text-figs. 4 and 5), but was difficult to fix. However, when left to themselves the dance ended in an hour or rather less and development then proceeded. After a further 12-24 hours the cyst would burst and liberate ripe spores—the average time for this appeared to be 18 hours—so that the approximate time for the development of ripe spores would be 30 hours from the extrusion of the cyst from the host. The spores ($7-8\mu$ in diameter) are slightly flattened at the poles. At first the equatorial ray-like processes (nearly 10μ long) wrap round the lower poles of the spores (Text-fig. 8) but as they float away these processes gradually extend themselves, appearing from above like the rays of the sun (Text-fig. 9).

The episporal processes are shown up very clearly by staining with Steven's blue-black ink, as has often been found before (Pixell Goodrich, 1929). They start to develop (Text-fig. 6) while the synkaryon is still resting and are apparently full length at the 4-nuclear stage (Text-fig. 7).

The 8 sporozoites (Text-fig. 10), each about 6μ long, seem to escape through an operculum, but we have not succeeded in observing this process either in the fore gut or outside. There is a residuum of large refringent granules.



TEXT-FIG. 4. Male gamete with nucleus and flagellum.

TEXT-FIG. 5. Zygote with synkaryon and flagellum.

TEXT-FIG. 6. Sporocyst from above with resting nucleus and developing episporal rays.

TEXT-FIG. 7. The same with 4 nuclei and 6 rays.

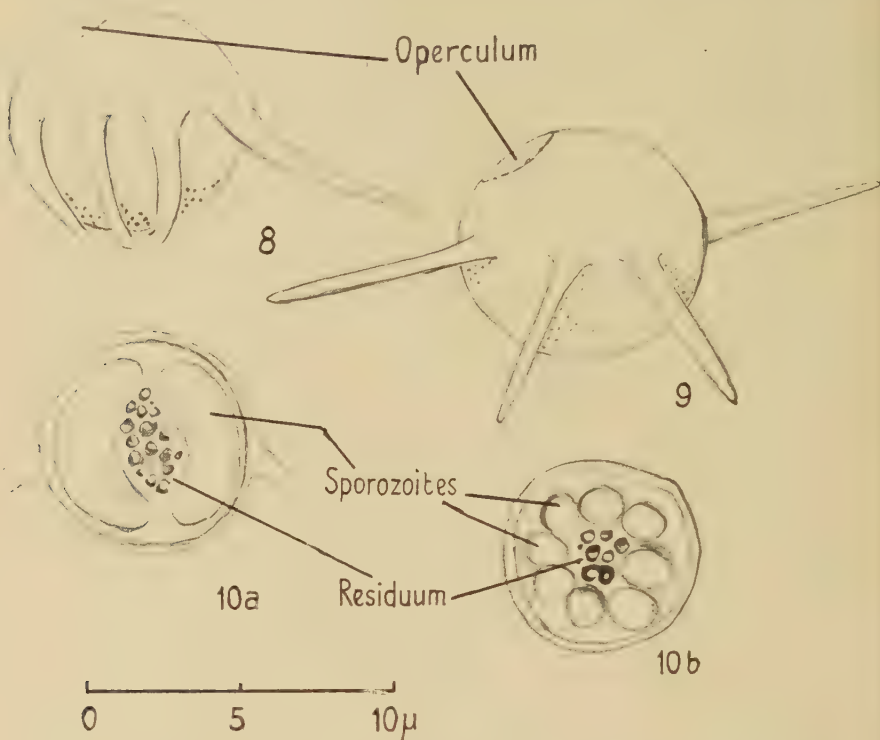
Figs. 4-7 fixed Schaudinn's solution and stained iron haematoxylin.

Rotundula gammari (Diesing, 1859)

This short somewhat rotund polycystid gregarine (Text-fig. 3) has been even more difficult to fit into any genus so far described and to prevent confusion the name *Rotundula* has been given. Superficially the trophozoite, of course, does show some resemblance to such members of the genus *Gregarina* as *G. ovata*, &c. However, it differs fundamentally in having no

large cyst with sporoducts through which the characteristic adhering spores emerge in long chains.

The generic difficulty arose chiefly from the fact that in 1908 Mavrodiadi gave a preliminary note in a small Russian paper of a polycystid gregarine



TEXT-FIG. 8. Spore just emerged from cyst with 5 of the episporal rays still folded underneath it.

TEXT-FIG. 9. Spore with expanded rays—drawn from one side.

TEXT-FIG. 10. Optical sections of spores (a) longitudinal, (b) transverse—showing sporozoites and residual protoplasm.

Figs. 8–10. Spores of *Heliospora longissima* drawn from the living.

in *Balanus* to which he gave the name *Cephaloidophora*. His figures (13) of an elongated spore and (7–11) of sporozoites and trophozoites growing deeply embedded in the tall epithelial gut cells of the *Balanus* are quite clear. In spite of this Mercier (1911) called a gregarine of the Caridine from Nancy *Cephalodophora cuenoti*. It had no intracellular stage and the next year (1912b) he removed it from Mavrodiadi's genus, saying (p. xliii, footnote): 'Je ne connais pas le mémoire original de Mavrodiadi écrit en russe.' He then called it *Uradiophora*, a genus proposed by him (1912b and c) to which I have referred above (pp. 27–8) as being an ill-defined genus to which Poisson (1921b) attached the long thread-like gregarine of *G. pulex*.

Unfortunately, in the intervening years, other gregarines were crowded into the genus *Cephaloidophora* (see Watson Kamm, 1922), many of them apparently having no better claims than Mercier's to be there, and one cannot help suspecting that some of the authors, like Mercier, had not studied Mavrodiadi's Russian paper.

In 1911 Léger and Duboscq described a polycystid gregarine from *Gammarus marinus* at Roscoff which is very like this *G. pulex* parasite *R. gammari*. Strangely enough this well-described parasite was also placed in Mavrodiadi's genus under the name of *Cephaloidophora maculata* after being removed from *Frenzelina* which was found to be preoccupied.

I should like to propose that it be changed to *Rotundula maculata* (L. and D.), and it would then be the type species of this genus, only differing apparently in having a marine host and possibly some of its young stages intracellular.

Rotundula n.g. Generic characteristics: Septate (polycystid) gregarines tending to rotundity, mostly free in the lumen of the gut but they may be temporarily attached to or between the epithelial cells.

Epimerite button-like and persistent.

Protomerite rounded with a nucleus in young stages.

Precocious association.

Cyst oval or round: wall covered with a gelatinous layer: no sporoducts.

Spores spherical or subspherical, small.

Specific characteristics of *R. gammari* (Diesing, 1859) from gut of *G. pulex*.

Everything about this gregarine is more or less round—a button-like epimerite, which, however, flattens out before encystment, the protomerite is almost spherical, vacuolated in the living and has 1 or sometimes 2 or 3 chromatic granules (nuclei) which, however, generally disappear before encystment.

The deutomerite may become almost spherical before encystment. Its nucleus is spherical and contains 1 large spherical karyosome. Cysts 40–50 μ , not adhesive. Spores small, more or less spherical with a faint equatorial suture; 8 sporozoites and a refringent residuum.

Very small specimens, from 6 μ long, have been seen free in the lumen or mixed with the cellular debris cast off with the peritrophic membranes from mid-gut and there is no doubt that they associate very early. I have seen a specimen lying between the shallow epithelial cells as shown in Léger and Duboscq's fig. V (1911). One would expect that the epimerites might cling to cells in this way as they do to the deutomerites of other specimens to form syzygies. In fact this does not appear to be a real intracellular stage as these distinguished protozoologists seem to assume.

A pair of trophozoites have often been seen folding over one another and encysting towards the end of the mid-gut. When eliminated the cysts are more difficult to find in the water than those of *H. longissima*.

They are, for one thing, smaller—seldom more than 50 μ —and they have an outer gelatinous wall which swells in water so that the cysts become about

the same density as the water and probably remain suspended: also they are not adhesive. The development when we have been able to follow it out appears to be just like that described by Léger and Duboscq (1911, fig. VI) for *C. maculata*. The gametes are roundish, the male, barely 4μ in diameter, having a fine flagellum. The spores are more or less spherical, only 5 or 6μ in diameter: they have a fine equatorial suture along which no doubt splitting takes place in the ripe spore and liberates the 8 small sporozoites, but we have not seen these escape.

Rotundula can be distinguished from *Cephaloidophora* in having a rounded not elongated spore and by most of its stages being free in the lumen of the gut.

Poisson (1921a) named a gregarine he had found in *Echinogammarus berilloni*, *Cephaloidophora echinogammari*, and proceeding to the observation of the gregarines of *G. pulex* (1921b) thought he discovered the same species there and made the curious suggestion that this was only present in *G. pulex* when this shrimp was living in close association with *Echinogammarus*.

This cannot possibly be true, for in this Oxford region, where we have studied *Gammarus* for some 25 years, there is no *Echinogammarus* nor other species of Gammarid than *G. pulex* (we are indebted to Charles Elton for confirming this). It may be infected with either or both the gregarines described here, viz. *H. longissima* (Siebold) or *R. gammari* (Diesing). No satisfactory record of any other than these two gregarines has been found throughout the 100 years that they have been studied. Therefore, if *Echinogammarus* contains the same parasite, it must be the species named *G. gammari* by Diesing in 1859.

In *Orchestia bottae*, recently found (Cain and Cushing, 1948) in numbers on the banks of the Thames here, there is an even more rotund gregarine which resembles *Rotundula* in its vegetative stages, but we have not studied its development. Poisson and Remy (1925) called it *C. orchestia*. It seems likely that this, as well as *C. echinogammari* Poisson, will be found to fit into the genus *Rotundula* as well as Léger and Duboscq's *C. maculata*.

In fact, the gregarines of most Amphipods will, I suspect, be found closely related to these long-known parasites of *G. pulex*.

I thoroughly agree with Poisson (1924, p. 247) that most of the gregarines of Crustacea require revision and their gametes and spores to be carefully studied: the cysts, as he remarks, are often difficult to collect. However, it cannot be too strongly emphasized that the important stages contained in them must be known before their proper systematic position can be determined.

In conclusion, I should like to express my thanks to Professor A. C. Hardy, F.R.S., who has put every facility at my disposal for continuing my work in his Department.

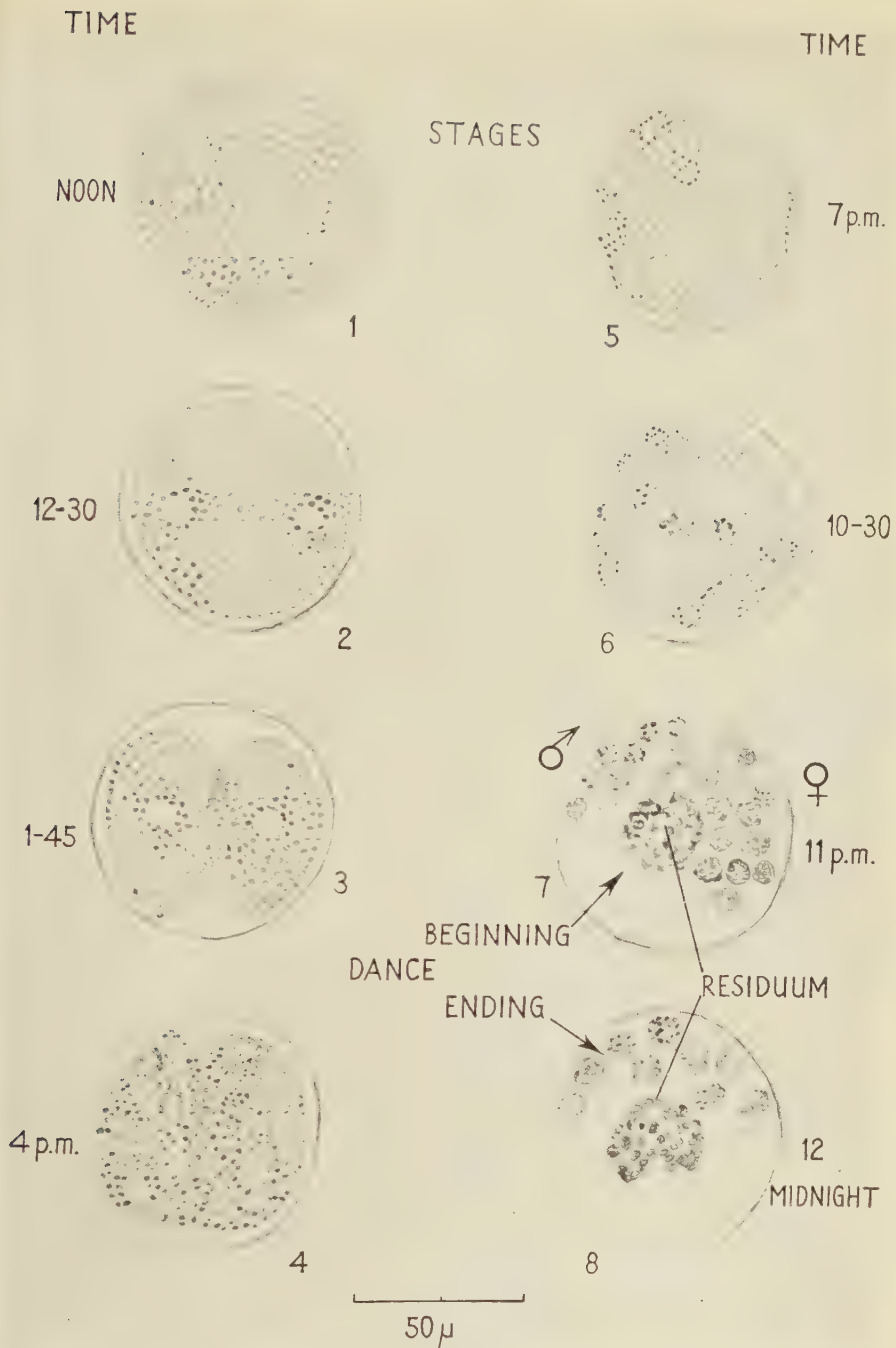
SUMMARY

From this study of the unknown stages in the life-histories of the two polycystid gregarines, which have been known for just 100 years in *Gam-*

marus pulex L., it is concluded that the long thread-like one should be *Heliospora longissima* (Siebold) and the short rounded one *Rotundula gammari* (Diesing), and that some other Amphipods have the same or similar gregarines.

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CYSTS AT VARIOUS STAGES

1. Cyst at extrusion (noon, on this occasion).
- 4-6. Cysts with nuclear division proceeding.
7. Cyst with ♂ and ♀ gametes—only a few of these are shown—sometimes the cyst is so full that only slight movement can be detected before syngamy.
8. Cyst showing a few zygotes moving round the residual protoplasm.

Studies on Abnormal Mitosis induced in Chick Tissue Cultures by Mustard Gas ($\beta\beta'$ -Dichlordiethyl Sulphide)

BY

A. F. W. HUGHES

(*Sir Halley Stewart Fellow*)

AND

HONOR B. FELL

(*Foulerton Research Fellow, Royal Society*)

(*From the Strangeways Research Laboratory, Cambridge*)

With three Plates

INTRODUCTION

THAT mustard gas causes structural changes in chromosomes was first shown indirectly by the important genetical experiments of Auerbach and Robson (1944, 1946, 1947), who found that by exposing adult male *Drosophila* to mustard-gas vapour a wide range of mutations was produced. Direct cytological evidence was provided by Koller (1947), who demonstrated various types of chromosomal abnormality in the pollen grains of *Tradescantia* after treatment with different concentrations of the vapour. These observations have recently been elaborated and extended by Darlington and Koller (1947).

In work done during the war for the Chemical Defence Research Department of the Ministry of Supply, Fell and Allsopp (1948) noticed profound disturbances of the mitotic process in the cells of tissue cultures growing in a medium containing 5–100 γ /c.c. of mustard gas. Later they found similar abnormalities in the regenerating epidermis of mice treated with repeated applications of minute quantities of the agent. Mitotic abnormalities have also been described by Gillette and Bodenstein (1946) and Bodenstein (1947) in amphibian embryos treated with a nitrogen mustard compound.

Recent optical developments have made it possible to study cell division in living material in much greater detail than hitherto. Hughes and Swann (1948) investigated the anaphase movement in cultures of normal chick osteoblasts, using both phase-contrast and polarized-light microscopy, and are accumulating more information about the mitotic spindle by these methods. It seemed desirable to extend this work with similar observations on different types of abnormal mitosis induced by chemicals and other agents, in the hope that the results might shed further light on the physiology of normal cell division.

The extensive literature on the pathology of mitosis has been reviewed by Politzer (1934). Abnormal mitoses in tissue cultures have been produced by

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very varied experimental means. Among the most notable contributions to this subject are the beautiful direct observations of reversible changes in living mitotic cells *in vitro* made by M. R. Lewis (1923, 1933*a* and *b*, 1934). The effect on dividing cells in culture of many different chemicals has been studied: various acids (M. R. Lewis, 1923; Bauer, 1923), reduced neutral red (M. R. Lewis, 1923), carbon dioxide (Mottram, 1928), ether (Kemp and Juul, 1930; Rosenfeld, 1932), potassium iodide (Razzesi, 1932), ammonia (Rosenfeld, 1933), auramine, urethane, methyl sulphonal, sodium cacodylate, colchicine and some of its derivatives, quinine, atropine, aconitine (Ludford, 1936), and of certain carcinogens and related hydrocarbons (Hearne Creech, 1939). The action of heat (Kemp and Juul, 1930; M. R. Lewis, 1933*a*), of hypotonic culture medium (M. R. Lewis, 1934), and of radiation (Strangeways and Oakley, 1923; Strangeways, 1924*a* and *b*; Lasnitzki, 1943; and others) have also been investigated. Abnormal mitosis may be produced merely by the addition of stale plasma to the culture medium (Strangeways, 1924). Cinema films of pluripolar mitosis *in vitro* have been made by W. H. Lewis (1932) and by G. Gey (1947).

The observations described in the present paper refer to cells growing in a medium containing low concentrations of mustard gas (pure $\beta\beta'$ -dichlorodiethyl sulphide). The results were obtained partly by the analysis of cinema films of the living cells made by phase-contrast microscopy, and partly from the cytological study of fixed and stained cultures; these two methods of approach were found to be complementary, and each demonstrated features of abnormal mitosis not shown by the other.

A. H. was responsible for the cinematography and the quantitative data obtained from the films and H. B. F. for the tissue culture and the observations on fixed material. The general analysis of the films and their interpretation were the joint work of both authors.

MATERIAL AND METHODS

Tissue Culture. Fragments of the frontal bones from 11- to 12-day fowl embryos were cultivated in a mixture of equal parts of fowl blood-plasma and tissue extract made with Tyrode from 11- to 12-day chick embryos. The explants were grown in hanging drop preparations on $1\frac{1}{4}$ in. square No. 2 coverslips over $3 \times 1\frac{1}{2}$ in. hollow-ground slides.

The cultures were incubated for 2–3 days, by which time the original bone fragment had become surrounded by a halo of migrating cells consisting mainly of osteoblasts. The tissue was then transferred to medium containing mustard gas in one of three concentrations: 12.5, 25.0, and 50.0 γ /c.c. The agent was introduced into the culture medium in the following way. A solution of mustard gas in absolute alcohol was prepared and a small quantity was added to a known volume of plasma in such a way that the plasma contained double the amount of the agent that was required for the final culture medium. A drop of this plasma solution was then placed on a No. 1 coverslip and mixed with an equal drop of embryo extract. The explant was transferred

to the mixture before it clotted, and for cinematography the coverslip was mounted on a special type of culture vessel; the application of phase-contrast illumination to tissue cultures and the culture chamber devised for this purpose have been described elsewhere (Hughes and Swann, 1948). Cultures required for fixation and staining were mounted on hollow-ground slides in the usual way. All the preparations were incubated for 24–48 hours before use.

In one experiment the explants were transferred to normal medium, incubated for 24 hours, then opened, and a drop of serum containing 50 γ /c.c. of mustard gas was deposited on the tissue. After this treatment the cultures were mounted on the special chambers mentioned above and examined either immediately or after 24 hours' further incubation.

Cinematography. The phase-contrast objective ($\times 95$) and condenser used for this work were supplied by Messrs. Cooke, Troughton & Simms, Ltd. (see Hughes and Swann, 1948). For the photomicrography of living cells in tissue cultures this apparatus should be used in conjunction with sensitized film of maximum contrast. This is particularly important for cells treated with mustard gas in which the contrast of the chromosomes is much below normal, probably owing to a diminished content of nucleic acid.

After many different types of film had been tried with the generous collaboration of the research staff of Messrs. Kodak, Ltd., a special 16-mm. negative of extremely high contrast was chosen, known as 'film for ciné-photomicrography'. The contrast in the photograph is greater than in the direct image, so that the cellular detail can be seen much better in a print than by direct observation through the microscope. The use of this film in the study of living cells will be described elsewhere.

The ciné records were studied exhaustively by projection in both directions, by examination frame by frame, and by comparing paper enlargements of selected series of frames. The sequences described below were analysed by means of all three methods. The rates of anaphase movement were obtained by measuring the distances between the daughter chromosome groups in successive frames projected on paper (Hughes and Swann, 1948).

Fixation and Staining. In most of the experiments some of the cultures were used for ordinary cytological study. They were fixed for 4–5 min. in Maximow's solution (10 parts Zenker's fluid: 1 part formol: 1 part 2 per cent. osmium tetroxide) freshly prepared for each occasion, washed overnight in distilled water, then treated with alcoholic iodine and washed with 70 per cent. alcohol. Some of the cultures were stained by Feulgen's method. Others were hydrolysed for 8 min. at 60° C. as for the Feulgen technique and then stained for 10–15 min. in well-ripened Ehrlich's haematoxylin; this method gave a very clear picture of the cells and of the chromosome structure and was particularly suitable for photography; the distribution of the stain was precisely the same as with the Feulgen technique. Preparations stained with haematoxylin without previous hydrolysis were much inferior in clarity to the hydrolysed specimens. The stained cultures were dehydrated, cleared, and mounted whole in Canada balsam.

For certain purposes fixation for 3–4 min. in Zenker's fluid without acetic acid followed by hydrolysis and staining with Ehrlich's haematoxylin was useful. This method was particularly suitable for demonstrating the smaller micronuclei in the many multi-nucleate cells which mustard gas produced in the cultures (Pl. III, fig. 31); it also rendered the spindle of mitotic cells very distinct (Pl. III, fig. 30).

RESULTS

Normal Mitosis as seen by Phase-contrast Microscopy

In normal living osteoblast cultures the intermitotic cell (Pl. I, fig. 3) is much flattened and usually of triangular or spindle-shaped outline; the oval nucleus, which is rather paler than the cytoplasm, contains one or more irregular nucleoli which appear nearly black. In the cytoplasm are seen the dark mitochondrial filaments, many small granules, and the highly refractile fat globules. Both the cell and its contents are in continual slow movement.

When prophase begins the cytoplasmic processes are largely withdrawn, the nucleoli vanish, and faintly grey, diffuse chromosomes materialize throughout the nuclear area. At the same time the nuclear membrane disappears, but cytoplasmic inclusions remain outside the nuclear area until late anaphase. The chromosomes contract, become increasingly distinct, and assume a radial orientation in the plane of the coverslip (Pl. I, fig. 1a); they probably lie in this plane as the mechanical result of the flatness of the cell. Whether the spindle has already begun to form is not yet known, and since it is therefore uncertain whether this stage should be regarded as late prophase or early metaphase, we have termed it the radial stage.

As the cell becomes more nearly spherical, the chromosomes rotate from the plane of the coverslip to one at right angles to it, presumably under the influence of the spindle elements, but in normal cells the spindle is indistinct with phase-contrast microscopy and the details of its formation cannot be seen. During metaphase (Pl. I, fig. 1b) the chromosomes move to and fro in the equatorial region of the spindle with unsynchronized linear motion (W. H. Lewis, 1939; Hughes and Swann, 1948). Without warning the chromatids suddenly separate and pass quickly to opposite poles (Pl. I, fig. 1c, d). Details of the anaphase movement have been described by Hughes and Swan (1948).

About 3 min. after the beginning of anaphase, the granules and mitochondria of the surrounding cytoplasm bulge into the inter-zonal region and constriction into daughter cells begins. 'Bubbling' of the peripheral cytoplasm, spreading from the poles to the equator (cf. Chambers, 1938), may occur at any stage of mitosis but always becomes increasingly vigorous during telophase (Pl. I, fig. 1e). The contrast of the chromosomes falls during telophase so that usually nothing is clearly visible in the nuclear area until 12–20 min. after anaphase, by which time the nuclear membrane and nucleoli are present and the daughter cells are flattening (Pl. I, fig. 1f). A connecting thread of cytoplasm persists for a very variable time and then snaps.

The stages of normal mitosis as they appear in fixed and stained cultures are shown in Pl. III, figs. 12-17.

The Effect of Mustard Gas on Mitosis

I. General Effects on the Cells (Table 1)

In living cells grown in the presence of small quantities of mustard gas and examined by phase-contrast microscopy, the cytoplasmic structures and particularly the mitochondria are abnormally distinct, but, as stated above, the contrast of the chromosomes is subnormal. Owing to the high contrast of the negative used, however, the general form of the chromosomes can be distinguished in cinema films of the living cells, but suitably fixed and stained preparations are required for a more precise study of chromosome structure.

At the higher concentrations of mustard gas the volume of the cell, as judged by its surface area, is increased. The fat content, even after 48 hours' cultivation, is abnormally low at all three concentrations, and the cytoplasm may be free of all but the smallest lipid granules. These small lipid granules are seen to be in Brownian movement by direct observation, whereas in the normal cell movement can only be directly appreciated in a minority of the globules. From this it may be inferred that the water-content of the cells treated with mustard gas is abnormally high and the viscosity of the cytoplasm relatively low.

The degree of mitotic disturbance caused by the presence of mustard gas in the culture medium varies in the same culture from a slight deviation from the normal to great irregularity. The proportion of extreme abnormalities is naturally much larger at the two higher concentrations, but even at the 12.5 γ /c.c. level a few greatly distorted mitotic figures are seen (Pl. III, fig. 28).

Three main types of abnormal mitosis may be distinguished, which are bipolar, tripolar, and apolar, respectively; they will be considered in detail in the next section.

II. Observations on the Three Main Types of Abnormal Mitosis

1. *Bipolar Mitosis.* At the lowest concentration of mustard gas (12.5 γ /c.c.), some mitoses are nearly normal. This is illustrated by one of the ciné records:

Record 1 (Pl. I, fig. 2). In this film mitosis, and in particular the radial stage, is unduly prolonged (see Table II), but otherwise division proceeds normally.

The abnormal bipolar mitoses may be divided arbitrarily into two groups, in one of which (group A) the abnormality is much less extreme than in the other (group B); in life the dividing cells of group B are usually larger than those of group A.

Mitoses of group A are characterized by the delayed arrival of certain chromosomes at the equatorial plate or their failure to reach it (Pl. III, figs. 18 and 19), by lag at anaphase and telophase (Pl. III, figs. 20-22), and by the formation of one or more micronuclei derived from the lagging chromosomes in addition to two daughter nuclei of nearly normal size (Pl. III, figs. 23 and 24).

TABLE I. *Summary of Observations, mainly from Cinema Records, of Mitotic Abnormalities produced by Mustard Gas*
 N. = normal R.I. = record incomplete

	Nearly normal	Bipolar		Tripolar	Apolar
		A	B		
Number of examples recorded by cinema	12.5 v/c.c.: 3 25.0 v/c.c.: 2 50.0 v/c.c.: 1 (in serum)	12.5 v/c.c.: 3	25.0 v/c.c.: 3 50.0 v/c.c.: 1 50.0 v/c.c.: 2 (in serum)	25.0 v/c.c.: 2	25.0 v/c.c.: 1 50.0 v/c.c.: 3
Illustrations	..	Pl. I, figs. 2, 4, 5 Pl. III, figs. 18-24	Pl. I, fig. 6 Pl. II, fig. 8 Pl. III, figs. 25-29	Pl. II, figs. 9, 10 Pl. III, fig. 30	Pl. I, fig. 7 Pl. II, figs. 11 Pl. III, figs. 31, 32
Radial stage	Duration	4 cells: N. 2 cells: R.I.	Fig. no. 2, 5	Fig. no. 10	Fig. no. 11
	Radial movement	2 cells: prolonged 1 cell: R.I. 1 cell: + 1 cell: N. 1 cell: R.I.	No record	1 cell: prolonged 1 cell: R.I. 1 cell: + 1 cell: R.I.	1 cell: prolonged 3 cells: R.I. 1 cell: + 3 cells: R.I.
Metaphase	Duration	3 cells: prolonged 1 cell: N. 2 cells: R.I.	Fig. no. 5	Fig. no. 9	Fig. no. 11
	Delay or failure in reaching metaphase plate Linear movements on spindle Condition of chromosomes	1 cell: + + N. N.	4 cells: prolonged 2 cells: R.I. Obscure in records. + 1 in fixed cells 1 cell: + + + 3 cells: + + + 1 cell: N. 1 cell: R.I. Incomplete contraction; pronounced beading	1 cell: no distinct metaphase 1 cell: + + 1 cell: + May be abnormally short and thick	No distinct metaphase Granules or distorted rods and filaments No recognizable anaphase
Anaphase	Movement of anaphase groups Lag	N. rate 2 cells: + 1 cell: -	N. rate 5 cells: + + 1 cell: -	1 cell: N. rate + +	No recognizable anaphase
Telophase	Type of cleavage	2 daughters	Fig. no. 6, 8	Fig. no. 9	No cleavage
	Rate of cleavage Cytoplasmic streaming	N. N.	About half N. rate 4 cells: + + 1 cell: + 1 cell: N. Several nuclei of various sizes	1 cell: 3 daughters 1 cell: at first 3 daughters, but 2 by secondary fusion About half N. rate + + + Several nuclei of various sizes	No cleavage + + + Many nuclei of different sizes
	Daughter nuclei	2 of N. size 1 cell: 2 of N. size	2 cells: 2 of N. size + micronuclei 1 cell: 2 of N. size		

Some of the chromosomes appear normal in stained preparations, but in others the nucleic acid charge is localized in granules, the intergranular material being nearly colourless when stained by Feulgen's method or by Ehrlich's haematoxylin (Pl. III, fig. 20). Some interesting features of these mitotic abnormalities are seen in two of the ciné records (Records 2 and 3).

TABLE 2. *Showing Phase Times in Minutes of Bi- and Tri-polar Mitoses*
N.N. = nearly normal

Treatment	Type of division	Pro-phase	Meta-phase	Beginning of anaphase to		Nucleoli in daughter cells	Record no. in text	Remarks
				Early cleavage	End of cleavage			
8 controls	Normal	..	c. 4	2'3-5'0	3'5-6'0	11-20	..	
12'5 γ /c.c.	N.N.	> 2'4	9'4	3'6	7'0	13'2	..	
"	N.N.	..	..	3'0	6'8	13'4	..	
"	..	..	..	3'8	6'6	10'0	..	
25'0 γ /c.c.	N.N.	..	> 6'7	3'4	6'2	9'1	..	
"	N.N.	> 4'7	9'9	3'7	6'1	9'4	..	
50'0 γ /c.c. (serum)	N.N.	> 4'2	21'4	3'8	9'4	11'4	..	Nucleoli still present when record begins
12'5 γ /c.c.	A	> 36'6	6'4	4'8	9'6	18'2	1	
"	A	..	> 7'2	5'8	8'4	13'0	2	
"	A	> 12'6	10'6	1'8	6'8	12'4	3	
25'0 γ /c.c.	B	12'0	..	3'9	5'9	7'8	..	
"	B	..	16'2	4'1	9'5	10'8	4	
"	B	..	13'0	3'2	5'5	13'9	..	
50'0 γ /c.c. (serum)	B	..	10'8	3'8	7'0	12'0	..	
"	B	..	> 135'0	3'3	6'2	17'2	5	
25'0 γ /c.c. (serum)	Tri-polar	..	> 1'8	5'0	9'5	10'7	6	

Record 2 (Pl. I, fig. 4). This cell shows a simple lag of chromosomes at anaphase within the spindle area; it finally divides into two daughter cells, one of which contains a single nucleus, while the other forms one nucleus of nearly normal size and two micronuclei.

Record 3 (Pl. I, fig. 5). The cell is in prophase when the film begins. Most of the chromosomes pass to the equator in the normal way, but two fail to join the others and remain near one pole of the spindle (Pl. I, fig. 5*b*). Within 2 min., however, the tardy chromosomes are drawn to the equator (Pl. I, fig. 5*c*) and soon afterwards anaphase begins. Several chromosomes on each side of the metaphase plate do not divide and separate with the rest, but lag behind at the equator (Pl. I, fig. 5*d*). As the peripheral cytoplasm bulges into the interzonal region preparatory to cleavage, the lagging chromosomes from each side are pushed towards each other and are finally brought together by the equatorial constriction (Pl. I, fig. 5*e*); this lateral form of lag will be discussed in more detail below. After anaphase the fate of the laggards is obscured by the bubbling of the cytoplasm. Eventually one daughter cell is seen to contain a single large nucleus and the other one large nucleus and a micronucleus.

The divisions of group B are much more abundant at the higher concentrations than at the 12.5 γ /c.c. level and the mitotic abnormality is an exaggeration of that described above in group A. The spindle seems relatively normal, but in stained preparations the chromosomes appear very deficient in nucleic acid and have not contracted properly; there is very pronounced lag at anaphase and the daughter cells contain more micronuclei than are formed in the divisions of group A.

In prophase (examined in fixed preparations only) the filaments are somewhat vaguely defined and stain lightly as compared with the normal; they often vary in thickness along their length and are sometimes beaded; they may be unevenly distributed in the nuclear area and in places entwined to form long, tangled, granular skeins (Pl. III, cf. figs. 12 and 25). At metaphase (Pl. III, figs. 26 and 27) the chromosomes are seen to vary enormously in size and appearance, some being minute granules, others long, beaded, and often attenuated filaments; in Feulgen preparations, the beads on the long chromosomes are Feulgen-positive while the rest of the thread is nearly colourless, indicating severe nucleic acid deficiency. Other chromosomes have an irregular outline and the Feulgen-positive material is aggregated in lumps here and there on the nearly unstained thread. Some chromosomes may fail to reach the equatorial plate and lie in the cytoplasm.

In many of the cells at metaphase most of the chromosomal material is aggregated into one or more large tangled skeins (Pl. III, figs. 26 and 27) similar to, but usually larger and more compact than, those sometimes seen at prophase (Pl. III, fig. 25). The filamentous structure of these masses varies in distinctness in different cells; in some they are quite clearly composed of entwined, beaded threads, while in others they appear as almost homogeneous bodies. These masses may lie on each side of the spindle at the equator or freely in the cytoplasm (Pl. III, fig. 27). Sometimes the large chromatinic masses move to the surface of the cell, where a strictly localized bubbling of the cytoplasm takes place (Pl. I, fig. 6a-c; Pl. III, fig. 27).

Anaphase (Pl. III, fig. 28) is characterized by a pronounced equatorial lag, usually of several chromosomes, though the two main daughter groups pass to the poles at the normal rate (Text-fig. 1). This failure of individual chromosomes to move normally to the poles of the spindle is of two types: a simple median lag, usually of small chromosomes, within the spindle, and a lateral lag, to which reference has already been made, which is associated with a somewhat diffuse structure of the metaphase plate, especially when the chromosomes are incompletely contracted. In the latter types, in which the equatorial plate is disproportionately large for the spindle, the long lateral chromosomes and sometimes large chromosomal masses (Pl. III, fig. 28) fail to move during anaphase and remain flanking the interzonal region, the interior of which is clear except for the small chromosomes undergoing median lag (Pl. II, fig. 8b-e).

When cleavage begins, constriction of the interzonal region pushes the mitochondria and the lateral lagging chromosomes into an axial position, mid-

way between the groups of daughter chromosomes; here they remain, until the completion of cleavage incorporates them in one or other of the daughter cells where they form micronuclei (Pl. II, fig. 8*e-g*). Such lagging filamentous chromosomes often produce a bridge of chromatin uniting the two daughter cells, and expanding at either end into an oblong or pear-shaped nucleus. Sometimes this bridge is surprisingly long and attenuated (Pl. III, fig. 29) indicating considerable plasticity of the chromosomes.

The multinucleate condition of the daughter cells in group B is not always due entirely to the lagging chromosomes. In some cells the daughter chromosomes of the two anaphase groups are very loosely arranged (Pl. III, fig. 28) and instead of forming a single large nucleus, give rise to a nest of micronuclei; there is some evidence both from the films and from fixed material that there may be secondary fusion in such nests, so that one or more larger nuclei are later formed. It is probable that chromosomal material which fails to reach the spindle at metaphase (Pl. III, fig. 27) also forms micronuclei at telophase.

Most of the phenomena described above are illustrated by two of the film records (Nos. 4 and 5).

Record 4 (Pl. II, fig. 8). The cell is in metaphase when photography begins. During anaphase a group of chromosomes moves to each pole in the usual way, but several chromosomes, in some of which a beaded structure is very distinct, lag behind (Pl. II, fig. 8*c*). When the cell constricts at cleavage, chromosomes previously lateral to the spindle and possibly not incorporated in the metaphase plate, together with some of the lagging chromosomes mentioned above, are pushed into an axial position (Pl. II, fig. 8*d, e*). Thus at telophase there are three groups of chromosomes in course of nuclear reconstruction—one equatorial and two polar (Pl. II, fig. 8*f*). Each polar group forms a single oval nucleus of normal size and appearance which moves freely in the cytoplasm. The equatorial group, however, produces a bouquet of small pear-shaped nuclei in one daughter cell (Pl. II, fig. 8*g*), which are unable to move freely until liberated from their attachment to the interzonal strand by the completion of cleavage. Finally, one daughter cell is seen to be mononucleate, while the other contains one nucleus of normal size and several micronuclei derived from the interzonal group.

Record 5 (Pl. I, fig. 6). In this cell both the spindle and the chromosomes are very distinct. When the record begins, there are several large, irregular granular masses and small chromatinic granules and filaments distributed over the spindle (Pl. I, fig. 6*a*). The smaller structures display very active, unsynchronized linear movement up and down the spindle, but the larger masses drift outwards, apparently under the influence of cytoplasmic currents. The filaments alternately stretch and retract as they move up and down, and the larger chromosomal bodies continually change shape. Some of the larger masses, though lying in the cytoplasm, retain their connexion with the spindle, but the largest body moves right out of the spindle area and slowly works its way to the surface of the cell, where an active and strictly localized bubbling

begins (Pl. I, fig. 6a-c). A small cytoplasmic protuberance is formed at this point into which the chromosomal body passes (Pl. I, fig. 6c). Metaphase was enormously prolonged and anaphase did not take place until 135 min. after the beginning of observation.

The rate of anaphase movement is normal, but the groups of daughter chromosomes represent a relatively small proportion of the total chromatin content of the cell (Pl. I, fig. 6e). Much of the material is incorporated in the large irregular bodies described above; other chromosomal structures remain scattered near the poles of the spindle, never having reached the equatorial plate, while others again lag behind during anaphase. When cleavage begins both daughter cells show a spiral streaming of the cytoplasm which is much more active in one cell than in the other. Eventually this mitosis produces two daughter cells, each containing many medium-sized and small nuclei (Pl. I, fig. 6f, g).

2. *Tripolar Mitosis.* In tripolar mitosis there may be a typical triradiate metaphase (Pl. II, fig. 9a and Pl. III, fig. 30), or a recognizable metaphase may be entirely omitted, the cell passing straight from the radial stage to a tripolar anaphase and telophase (Pl. II, fig. 10). An example of both types of tripolar division was recorded by the cinema (Records 6 and 7).

Record 6 (Pl. II, fig. 9). A regular triradiate metaphase with active linear movement up and down the spindle axes is seen. Not all the chromosomes are incorporated in the spindle area, many being scattered throughout the surrounding cytoplasm. Suddenly the cell enters anaphase (Pl. II, 9b) and a group of chromosomes passes to each of the three poles, leaving a fourth group of laggards moving irregularly in the centre of the cell (Pl. II, fig. 9c). Meanwhile the outline of the cell becomes triangular and the cytoplasm begins to bubble. Bubbling increases in violence and the cell divides into three multinucleate daughters (Pl. II, fig. 9d-e).

Record 7 (Pl. II, fig. 10). This shows a much less regular tripolar division. An incomplete ring of chromosomes (Pl. II, fig. 10a) in active radial movement passes directly into a tripolar anaphase (Pl. II, fig. 10b), without forming a recognizable metaphase. All the chromosomal material seems to be in the form of granules; some are double and the two constituents are pulled apart at anaphase. Telophase, with violent bubbling and streaming of the cytoplasm, produces three daughter cells united by narrow bridges (Pl. II, fig. 10c), but before cleavage is complete two of the daughter cells reunite, so that finally one large and one smaller cell are formed, both of which are multinucleate (Pl. II, fig. 10d).

3. *Apolar Mitosis.* In mitosis of the apolar type there is no recognizable metaphase or anaphase. In some cells the chromosomal material is in the form of distorted filaments and rods, but in others most of it appears as granules (Pl. III, fig. 32), some of which are fairly large but others so small as to be only just visible. These dust-like particles are often arranged in radiating lines, but it is impossible to see whether they are discrete bodies or minute beads on a continuous unstained thread. Usually these cells also

contain one or more much larger chromatinic bodies, in some of which a closely tangled filamentous structure is distinguishable in fixed preparations while others appear as homogeneous globules (Pl. III, fig. 32). This modified radial stage may continue for a fairly long time, to be succeeded by a phase of nuclear reconstruction in which the scattered chromosomal material forms a host of nuclei of widely varying sizes (Pl. III, fig. 31). There is no cleavage of the cytoplasm.

Cinema films were made of three cells of this type, two of which are described below (Records 8 and 9):

Record 8 (Pl. II, fig. 11). When the film begins, radially arranged granules of chromatin are seen in active radial movement. Suddenly this scattered group of chromosomal bodies is churned round by a rapid spiral streaming of the cytoplasm which continues for a short time and then gradually subsides. The cell begins to flatten and a number of nuclei develop (Pl. II, fig. 11*d*). Finally, a single large multinucleate cell is formed (Pl. II, fig. 11*e*); one nucleus is much larger than the rest and appears to be the fusion product of several smaller nuclei.

Record 9 (Pl. I, fig. 7). In this cell the chromosomal material is very dispersed (Pl. I, fig. 7*a*), but two large lumps of chromatin are clearly seen at one stage (Pl. I, fig. 7*b*). At first the cell is fairly quiescent, then an active spiral movement of the cytoplasm, associated with violent bubbling, begins (Pl. I, fig. 7*b*); this commotion gradually dies away and a single cell results in which about 17 nuclei were counted in life (Pl. I, fig. 7*c*).

III. *Time Relations in Abnormal Mitosis* (Table 2).

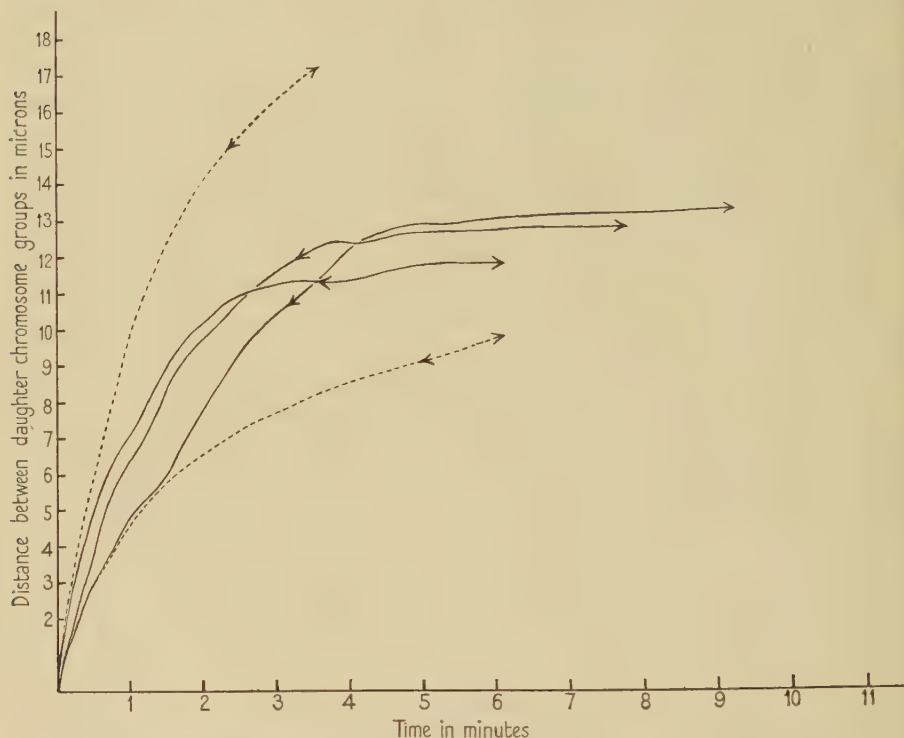
The duration of each mitotic phase in cells dividing under the influence of mustard gas was measured from the photographic records. The figures obtained, with comparable data from normal material, are presented in Table 2.

The most sharply defined stages in mitosis are the beginning of anaphase and the end of cleavage; the transition from the radial stage to metaphase is more gradual but can usually be estimated to within half a minute. Photography began either at prophase or metaphase, and the duration of the phase in which the cell was first observed is indicated as more than ($>$) the recorded time. The late phases of chromosome division are reckoned from the beginning of anaphase; the beginning of cleavage is defined as the constriction inside the cell of the interzonal region of the spindle or, when this is not visible, as the first appearance of an external cleavage furrow.

In general, mitosis is prolonged by the influence of mustard gas, and prophase (Pl. I, fig. 2) and metaphase (Pl. I, fig. 6) in particular may be greatly protracted. The maximum duration of a prophase that was known to be succeeded by a metaphase was >36.6 min. Twice we followed what appeared to be a prophase for 78 min. (12.5γ c.c. mustard gas) and 144 min. (50γ c.c.) respectively, but during these periods the cells did not enter metaphase and observation was discontinued.

The maximum duration of a metaphase that we have recorded was >135 min. (Pl. I, fig. 6), a period several times as long as that of any other metaphase that we have studied. Such an extreme prolongation seems to be exceptional.

Anaphase proceeds at the normal rate. Curves of anaphase movement plotted against time were obtained from our cinema records (Text-fig. 1);



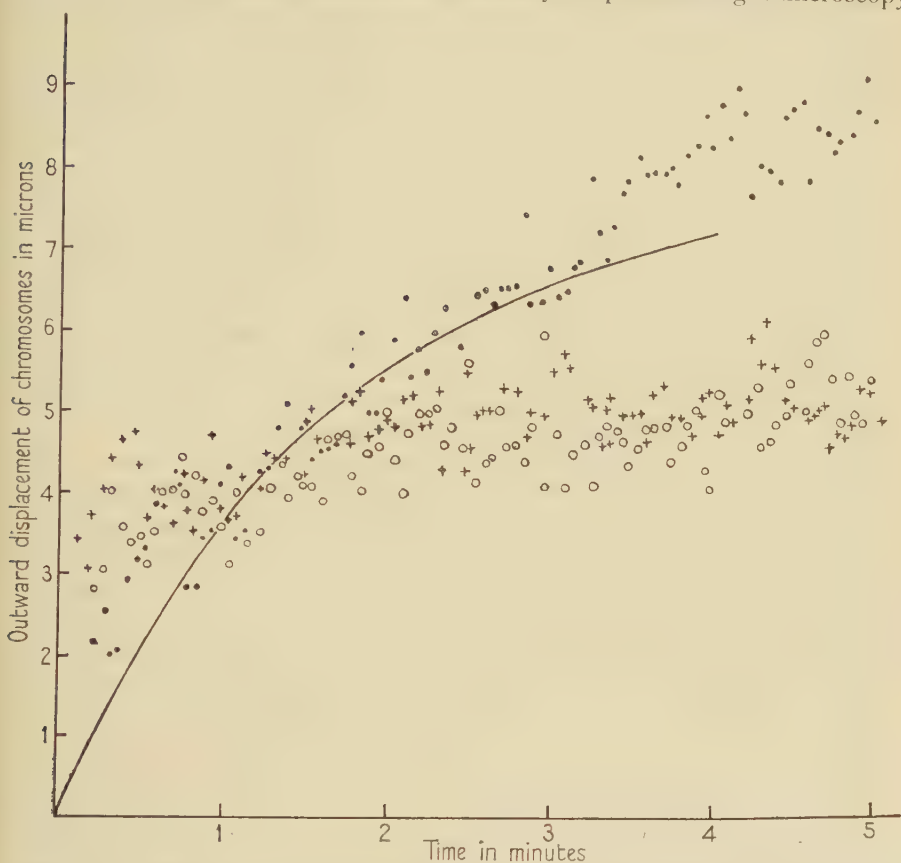
TEXT-FIG. 1. Anaphase curves of three bipolar mitoses from cultures treated with $50 \gamma/c.c.$ mustard gas, compared with curves (dotted) which represent the two extremes of a group of eight mitoses in normal osteoblasts. The beginning and end of cleavage is indicated.

when compared with curves from similar records of normal osteoblast cultures (Hughes and Swann, 1948) they are seen to fall within the normal range as regards rate of movement, distance traversed by the chromosomes, and general shape. At the higher dosage levels, however, the curves tend to move towards the lower limit of the normal range. The curves in the text-figure do not, of course, refer to the lagging chromosomes, which either remain stationary or move more slowly than the others.

Cleavage is somewhat, but not greatly, protracted, but the reconstruction of daughter nuclei, as indicated by the interval between the beginning of anaphase and the first appearance of nucleoli, is not greater than normal and sometimes may possibly even be less. Comparison with the corresponding period in the division of normal cells, however, is complicated by the fact that reconstruction of the daughter nuclei is less obscure in the

treated than in the untreated cells, so that possibly the formation of nucleoli is visible at an earlier stage in the abnormal mitoses.

A normal rate of chromosome movement at anaphase with a somewhat prolonged cleavage are also found in cells growing in normal medium, but subjected to the intense illumination necessary for polarized light microscopy



TEXT-FIG. 2. Measurements of the outward displacement of chromosomes in a tripolar mitosis in an osteoblast culture treated with 25 γ c.c. mustard gas (Record 6). The three symbols refer to each of the three half-spindles. The continuous curve is the average displacement of one group of daughter chromosomes in eight normal osteoblast mitoses.

(Hughes and Swann, 1948), or to γ -radiation, as well as in cultures treated with other chemicals (Hughes, unpublished).

As stated above, we have one cinema record of a fairly orderly anaphase movement in a tripolar mitosis (Pl. II, fig. 9). In this cell a group of chromosomes remains in the centre, and the distance between this central group and each of the three daughter groups during anaphase was plotted (Text-fig. 2). It will be seen that the rate of movement of each chromosome group in the tripolar anaphase corresponds roughly with that of a single daughter group in a normal bipolar anaphase.

DISCUSSION

The effects of mustard gas on mitosis *in vitro* are not specific. Such minor abnormalities as failure of one or more chromosomes to reach the equatorial plate, slight lag at anaphase, and the production of one or two micronuclei, are produced by many mildly unfavourable conditions. The more severe abnormalities described above most nearly resemble those resulting from irradiation (Strangeways and Oakley, 1923; Strangeways, 1924*b*; Lasnitzki, 1943), though they are also rather similar to those caused by ammonia (Rosenfeld, 1933).

Some of the phenomena we have described are essentially the same as those observed by Koller (1947) and Darlington and Koller (1947) in the pollen grains of *Tradescantia* treated with mustard gas, though mitosis seems to have attained a more extreme abnormality in the tissue cultures than in *Tradescantia*. Darlington and Koller were chiefly concerned with the breakage of chromosomes in relation to genetical problems, while our interest has been focused on the effects of the agent on the general physiology of cell division. These authors stress the close resemblance between the changes produced in their material by mustard gas and by X-rays.

Darlington and Koller describe and figure nucleic acid deficiency ('nucleic acid starvation', Darlington and Koller, 1947), similar to that seen in many of the mitotic figures in the treated tissue cultures; they also record imperfect contraction of the chromosomes; fragmentation, sometimes into small particles ('minutes' and 'subminutes'); failure or delay in reaching the metaphase plate ('errors of congression'); lag in anaphase, and the formation of micronuclei from chromosome fragments. They noted a correlation between centromere defects and errors of chromosome movement in *Tradescantia*, and it is probable that in the osteoblast cultures failure or delay of certain chromosomes to take part in the normal movements of metaphase and anaphase are due to defects in the attachment of the chromosomes to the spindle elements.

Whether chromosome breakage and reunion such as that seen in *Tradescantia* occurs also in our cultures is not known; we hope to extend our investigations to amphibian tissue cultures which are much more favourable for detailed cytological study than those of the chick.

Bodenstein (1947), in his interesting studies of the effects of a nitrogen mustard compound on amphibian development, describes cytological abnormalities in the ectoderm of *Amblystoma* embryos, which resemble those seen in the osteoblast cultures treated with mustard gas. He records the enlargement of those cells which normally divide actively, 'metaphases with irregularly arranged chromosomes' and anaphase lag; in the ectoderm, as in the tissue cultures, normal and abnormal mitotic figures occurred in close proximity. Multinucleate cells appeared in the ectoderm 7–8 days after exposure to the agent when mitosis had ceased; Bodenstein believes that the nuclei of these cells were formed by the fragmentation of enlarged interphase nuclei. In our material such cells were seen to arise by abnormal mitosis. There is

also strong cytological evidence that in mouse skin treated with repeated applications of mustard gas in very dilute solution the many multinucleate cells which appeared in the regenerating epidermis were the result of abnormal mitosis (Fell and Allsopp, 1948). While it is of course possible that the ectodermal cells of the *Amblystoma* embryos reacted rather differently from those of mouse epidermis or of chick osteoblast cultures, it would be interesting to know whether anything similar to the apolar mitosis described above (cf. Pl. I, fig. 7, Pl. II, fig. 11, Pl. III, fig. 32) was present in the amphibian tissue. The interpretation of such grossly abnormal mitotic figures is difficult in material which precludes the direct observation of the living cells.

Precisely how mustard gas disturbs the physiology of dividing cells is not clear. The agent is known to have several biochemical effects: on proteins in general (Banks *et al.*, 1946), on nucleo-proteins (Berenblum and Schoental, 1947), and on carbohydrate metabolism through the inactivation of hexokinase (Dixon and Needham, 1946). Which of these effects operate in living cells exposed to very low concentrations of the agent, and to what extent, remains to be discovered.

Study of the cinema records emphasizes the fact that the various phenomena of mitosis are not rigidly linked to each other, and that in the same cell one process may be grossly distorted, or even omitted, while another proceeds almost normally. Thus the chromosomes may be extremely abnormal in structure, as in group B of the bipolar divisions, and yet at anaphase a considerable proportion of them move to the poles of the spindle at the normal rate: in one of the tripolar mitoses described above there is no metaphase, but the cell is able to pass directly from the radial stage into a tripolar anaphase; in the apolar mitoses not only metaphase but anaphase and telophase also are omitted, but nevertheless the general cytoplasmic upheaval normally characteristic of these phases takes place and the scattered and distorted chromosomal material is able to form nuclei.

These observations suggest that in mitosis there are parallel series of reactions, in the chromosomes, in the spindle, and in the cytoplasm, which in normal division are closely co-ordinated but which under the influence of mustard gas and other agents may partially disengage and to some extent proceed independently of each other.

The authors are indebted to the Royal Society and to the Medical Research Council by whom the expenses of the investigation were defrayed. They also wish to express their thanks to Mr. V. C. Norfield for the photomicrographs on Pl. III and to their assistant Mr. L. J. King for his help with the tissue culture. They are also indebted to Dr. H. McCombie of the Department of Chemistry, Cambridge University, who kindly provided the mustard gas and to Dr. C. B. Allsopp for preparing the stock alcoholic solution.

SUMMARY

1. The cytological effects produced in cultures of embryonic fowl osteoblasts by low concentrations of mustard gas in the nutritive medium have

been studied both in the living cells by means of cinematography and phase-contrast microscopy, and in fixed and stained preparations.

2. Under the influence of mustard gas the movements of the cell and its contents are exaggerated and, especially at the higher concentrations, its water-content appears to be increased.

3. The treated cultures contain many abnormal mitotic figures which are more abundant and more distorted at the higher concentrations.

4. Three main types of abnormal mitosis have been observed:

(i) *Bipolar*. Group A: these cells are characterized by failure or delay of certain chromosomes in reaching the equatorial plate, by lag at anaphase and telophase, and by the formation from the lagging chromosomes of one or more micronuclei, in addition to two nuclei of normal size. Most of the chromosomes appear normal, but in some the nucleic acid charge is localized in granules. Group B: the mitotic abnormality is an exaggeration of that seen in group A. The spindle is relatively normal; the chromosomes fail to contract properly and have a beaded structure; some chromosomes may break up into small granules while others aggregate into large, granular, skein-like masses; there is an equatorial lag, usually of several chromosomes; multinucleate daughter cells are formed.

(ii) *Tripolar*. Two forms of tripolar mitosis have been observed: (a) a fairly regular triradiate metaphase plate was succeeded by a tripolar anaphase and the formation of three multinucleate daughter cells; (b) a recognizable metaphase was omitted, the cell passing straight from the radial stage (see p. 40) to a tri-polar anaphase and telophase.

(iii) *Apolar*. There is no recognizable metaphase, anaphase, or telophase; the chromosomal material has a radial orientation; in some cells it is in the form of distorted filaments and rods, while in others it appears as granules of different sizes. After a period of intense cytoplasmic turmoil the cell spreads out without cleavage and many small and medium-sized nuclei have been formed from the diffuse chromosomal material.

5. In the abnormal cells the duration of mitosis is prolonged; at the higher concentrations prophase and metaphase may last for 2 hours or more; when a spindle is formed, some of the chromosomes move apart during anaphase at the normal rate; cytoplasmic cleavage may occupy 2–3 times the normal period; reconstruction of the daughter nuclei proceeds at the normal rate.

6. The cytological effects of mustard gas resemble those of irradiation.

7. The observations indicate that many phenomena of mitosis, though normally closely co-ordinated, under abnormal conditions can to some extent disengage and proceed independently of each other.

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DESCRIPTION OF PLATES

The figures in Plates I and II are of living cells photographed by phase-contrast microscopy. All were enlarged to the same magnification ($\times 1000$) from single frames of ciné records. In each series the times are reckoned from that of the first picture. The photographs in Plate III are of fixed and stained preparations and were taken by Mr. V. C. Norfield, head assistant at the Strangeways Research Laboratory.

PLATE I

Fig. 1a-f. Stages in the normal division of an osteoblast; a. radial stage; b. $8\frac{1}{2}$ min., end of metaphase; c. $9\frac{1}{2}$ min., early anaphase; d. 12 min., late anaphase; e. $13\frac{1}{2}$ min., telophase; general cytoplasmic bubbling; f. 22 min., reconstruction of daughter cells: nucleoli have appeared.

Fig. 2a-f. $12.5 \gamma/c.c.$ mustard gas in the culture medium. Bipolar mitosis. Prolonged prophase, division otherwise normal. a. radial stage; b. 32 min., early metaphase; c. $36\frac{1}{2}$ min., end of metaphase; d. 40 min., anaphase; e. $45\frac{1}{2}$ min., late telophase; f. $62\frac{1}{2}$ min., apparently normal mononucleate daughter cells.

Fig. 3. Normal intermitotic cell. Note the two irregular nucleoli in the large oval nucleus, and the very refractile cytoplasmic fat globules.

Fig. 4a-e. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A) showing chromosome lag in anaphase. *a.* end of metaphase; *b.* 1½ min., early anaphase; *c.* 3½ min., late anaphase; *d.* 8 min., telophase; *e.* 32 min., daughter cells: in the left-hand cell one large nucleus and two micronuclei are forming.

Fig. 5a-i. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A) showing delay of two chromosomes in reaching metaphase plate and lag in anaphase. *a.* prophase; *b.* 6½ min., early metaphase: chromosomes $\times$ not yet in equatorial plate; *c.* 17½ min., metaphase: $\times$ nearly on the plate; *d.* 24 min., early anaphase: $\times$ incorporated in right chromosome group, lateral lag of two small chromosomes (seen as small black rods at the equator); *e.* 27½ min., early telophase: lagging chromosomes being pushed into an axial position; *f.* 29½ min., late telophase; *g.* 35½ min., *h.* 44 min., *i.* 55 min., stages in nuclear reconstruction: in the left daughter cell a micronucleus develops as well as a normal nucleus.

Fig. 6a-g. 50 γ c.c. mustard gas added in serum (see p. 39). Abnormal bipolar mitosis (Group B) showing large, granular chromosomal masses, one of which ($\times$) is expelled from the spindle, and the formation of two multinucleate daughter cells. *a.* metaphase: $\times$ in spindle area; *b.* 5½ min., metaphase: $\times$ leaving spindle area; *c.* 45 min., metaphase: $\times$ in cytoplasmic bubble; *d.* 134 min., end of metaphase; *e.* 136½ min., anaphase; *f.* 139½ min., telophase; *g.* 153 min., multinucleate daughter cells: note wide range of nuclear size.

Fig. 7a-c. 25 γ /c.c. mustard gas. Apolar mitosis forming single multinucleate cell. *a.* diffuse chromosomal material: absence of spindle; *b.* 10 min., two chromosomal masses visible, violent cytoplasmic bubbling; *c.* 35 min., multinucleate cell.

PLATE II

Fig. 8a-g. 25 γ /c.c. mustard gas; abnormal bipolar mitosis (Group B) showing median and lateral anaphase lag, beading of the chromosomes and the formation of one mono- and one pluri-nucleate daughter cell. *a.* end of metaphase; *b.* 1½ min., early anaphase; *c.* 2¾ min., anaphase: lagging beaded chromosomes; *d.* 3¾ min., late anaphase: lateral lagging chromosomes being pushed into an axial position; *e.* 7½ min., telophase: lateral chromosomes now axial; *f.* 15 min., daughter cells: abnormal persistence of cytoplasmic bridge, in lower daughter cell, bouquet of micronuclei formed from lagging chromosomes; *g.* 20 min., daughter cells: one mono- and one pluri-nucleate.

Fig. 9a-g. 25 γ /c.c. mustard gas. Tripolar mitosis forming three multinucleate daughter cells. *a.* end of triradial metaphase; *b.* 1½ min., early anaphase; *c.* 4 min., late anaphase: some chromosomes left in the middle of the spindle area; *d.* 6½ min., telophase; *e.* 10 min., *f.* 16 min., *g.* 18 min., stages in the formation of three multinucleate daughter cells.

Fig. 10a-d. 25 γ /c.c. mustard gas. Irregular tripolar mitosis with no recognizable metaphase, forming two daughter cells. *a.* radial stage: incomplete ring of chromosomes; *b.* 10 min., tripolar anaphase; *c.* 14¾ min., tripolar telophase; *d.* 28 min., coalescence of two of the three daughter cells.

Fig. 11a-e. 50 γ /c.c. mustard gas. Apolar mitosis forming single multinucleate cell. *a.* radial stage showing ring of chromosomes; *b.* 36 min.; *c.* 40 min., expansion of cell processes; *d.* 100 min., a nest of nuclei have been formed, note the nucleoli; *e.* 115 min., cell fixed and stained, the nest of nuclei are clearly seen. ($\times 1300$)

PLATE III

All the photographs, except Figs. 29 and 31, were taken at the same magnification as that of Fig. 12. The cells shown in Figs. 30 and 31 are from preparations fixed in Zenker's solution without acetic acid; the rest are from cultures fixed in Maximow's fluid. Figs. 13, 14, 19, and 26 were made from preparations stained by Feulgen's method and the remainder from preparations hydrolysed as for the Feulgen technique and then stained with Ehrlich's haematoxylin.

Fig. 12. Normal prophase. ($\times 1700$.)

Fig. 13. Late normal prophase.

Fig. 14. Normal radial stage.

Fig. 15. Normal metaphase.

Fig. 16. Normal anaphase.

Fig. 17. Normal late telophase.

Fig. 18. 25 γ /c.c. mustard gas. Abnormal bipolar metaphase (Group A), showing delay of one chromosome in reaching the equatorial plate; note the beaded end of this chromosome.

Fig. 19. 25 γ /c.c. mustard gas. Abnormal bipolar metaphase (Group A), showing chromosome right outside the spindle area.

Fig. 20. 12.5 γ /c.c. mustard gas. Abnormal bipolar anaphase (Group A), showing chromosome lag. Note the beaded structure of some of the chromosomes indicating nucleic acid deficiency.

Fig. 21. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A); early telophase with single lagging chromosome.

Fig. 22. 12.5 γ /c.c. mustard gas. Abnormal bipolar telophase (Group A) with several lagging chromosomes.

Fig. 23. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A); late telophase with three lagging chromosomes in course of reconstruction into micronuclei.

Fig. 24. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A); daughter cells, one containing a single large nucleus and two micronuclei and the other one large and one small nucleus.

Fig. 25. 25 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group B); prophase (cf. Figs. 12 and 13) showing granular chromosomes of irregular shape and distribution; at the chromosomal material forms a granular, skein-like mass.

Fig. 26. 25 γ /c.c. mustard gas. Abnormal bipolar metaphase (Group B); most of the chromosomal material is included in two large, granular masses on either side of the fairly normal spindle. The chromosomes in the spindle area are very deficient in nucleic acid and irregular in form.

Fig. 27. 50 γ /c.c. mustard gas. Abnormal bipolar metaphase (Group B) similar to that seen in Fig. 26. This cell contains three large chromosomal masses, two of which are lateral to the spindle, while the third lies freely in the cytoplasm near the surface of the cell. Note the localized cytoplasmic bubbling in the neighbourhood of all three masses.

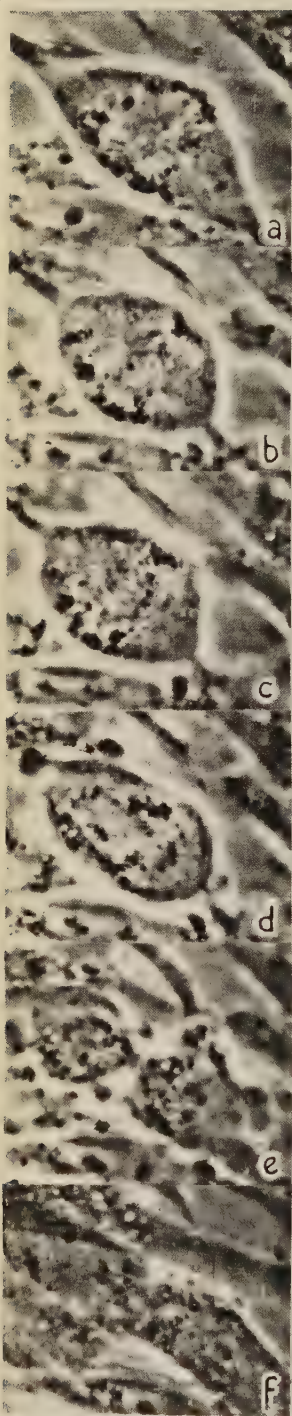
Fig. 28. 12.5 γ /c.c. mustard gas. Abnormal bipolar anaphase (Group B) showing two diffuse groups of daughter chromosomes and at the equator several beaded lagging chromosomes and large chromosomal masses.

Fig. 29. 25 γ c.c. mustard gas. Abnormal bipolar mitosis (Group B) showing multinucleate daughter cells united by a long chromatinic bridge, each end of which has expanded into a nucleus. ($\times 1150$.)

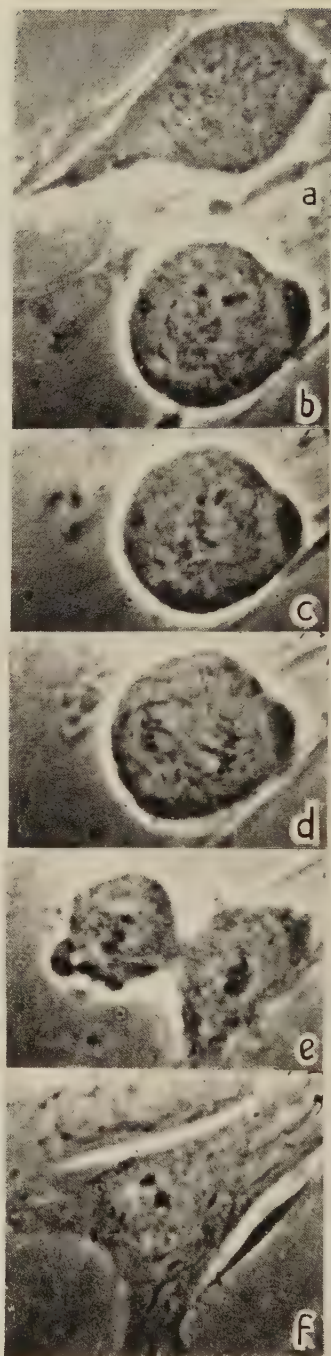
Fig. 30. 50 γ /c.c. mustard gas. Tripolar metaphase.

Fig. 31. 50 γ /c.c. mustard gas. Multinucleate cell derived from an apolar mitosis. ($\times 880$.)

Fig. 32. 50 γ c.c. mustard gas. Apolar mitosis showing radially arranged, granular chromosomes and homogeneous chromatinic body.



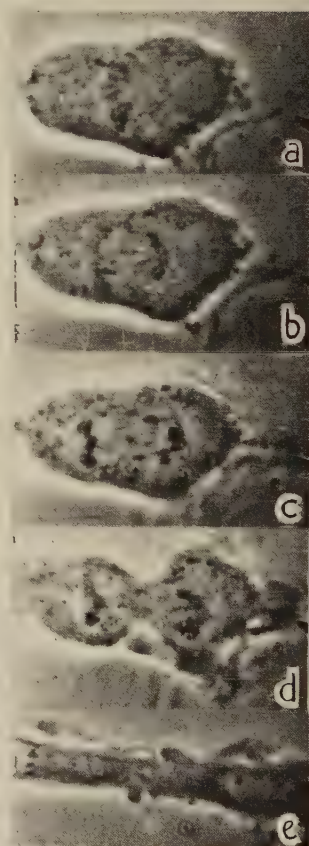
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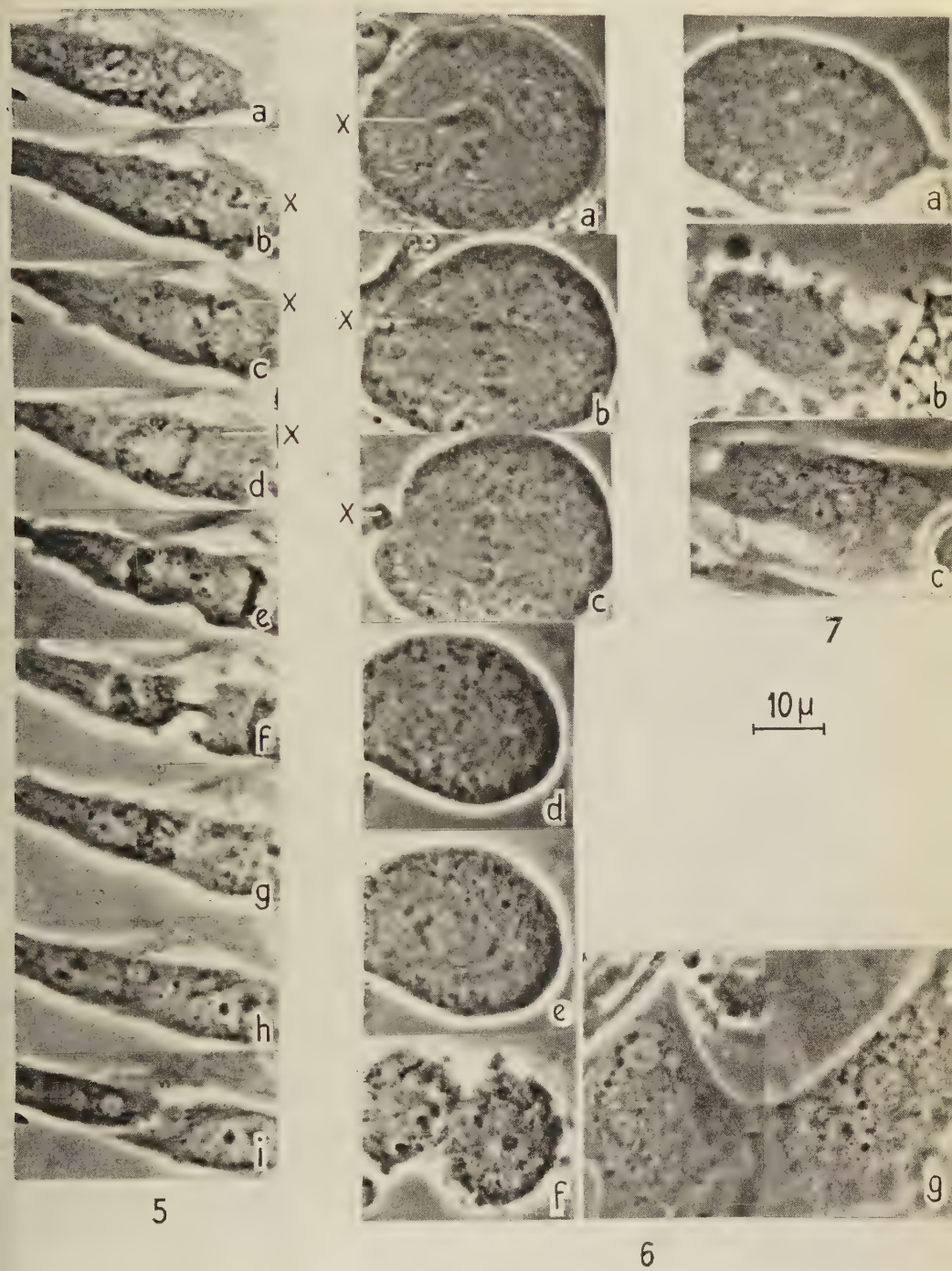
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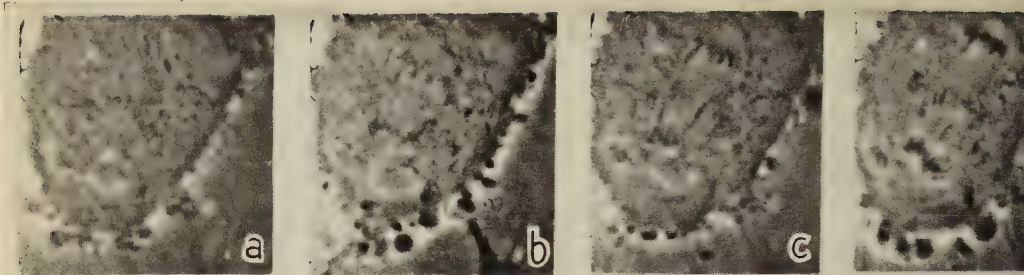


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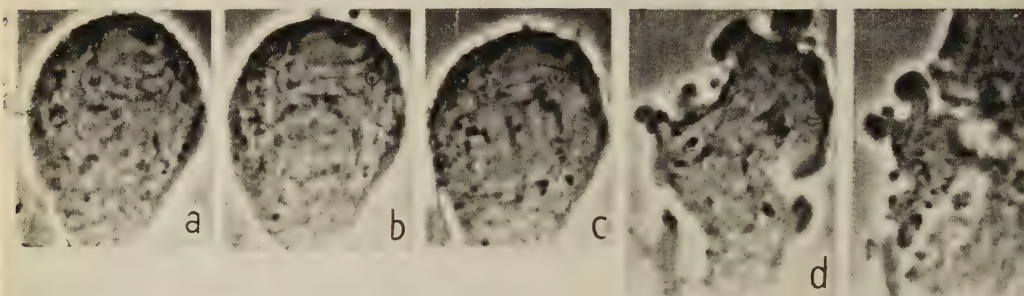


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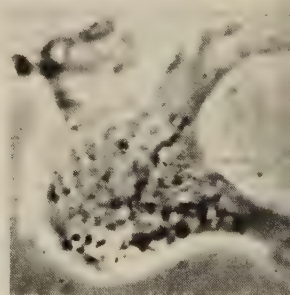
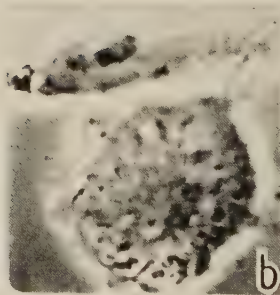
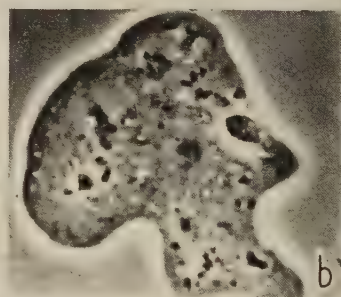
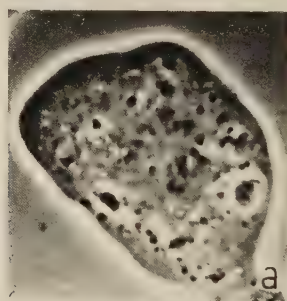


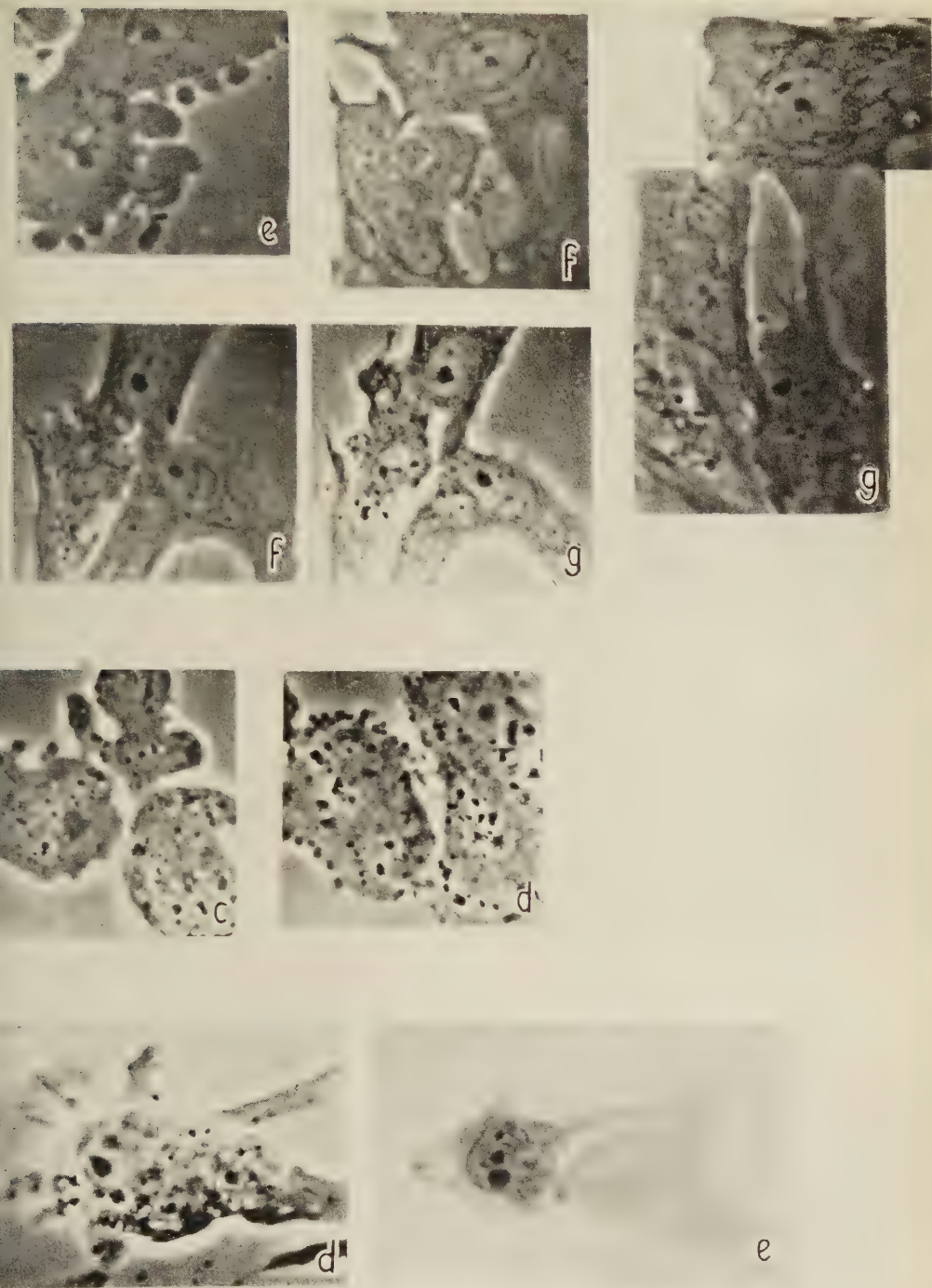


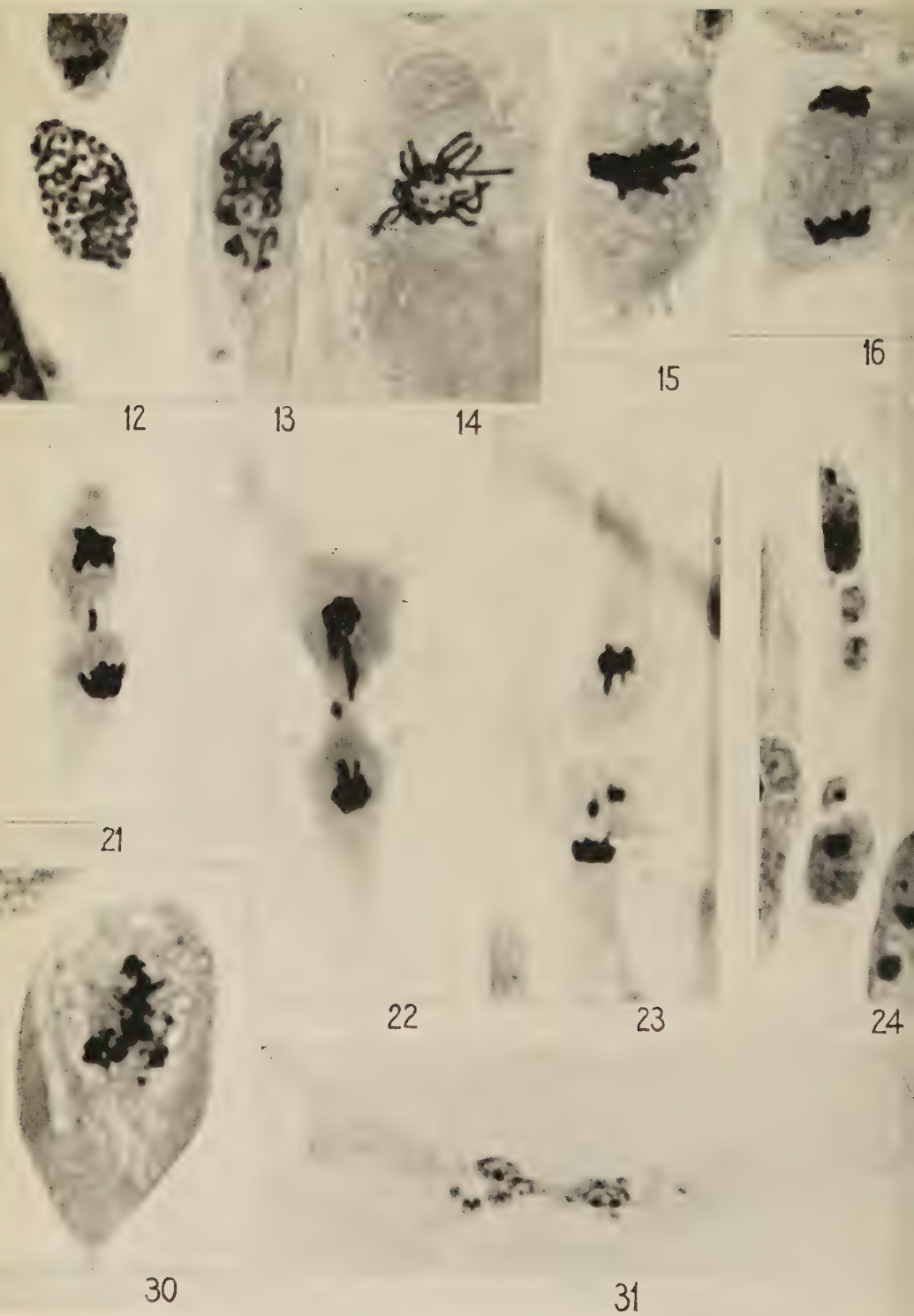
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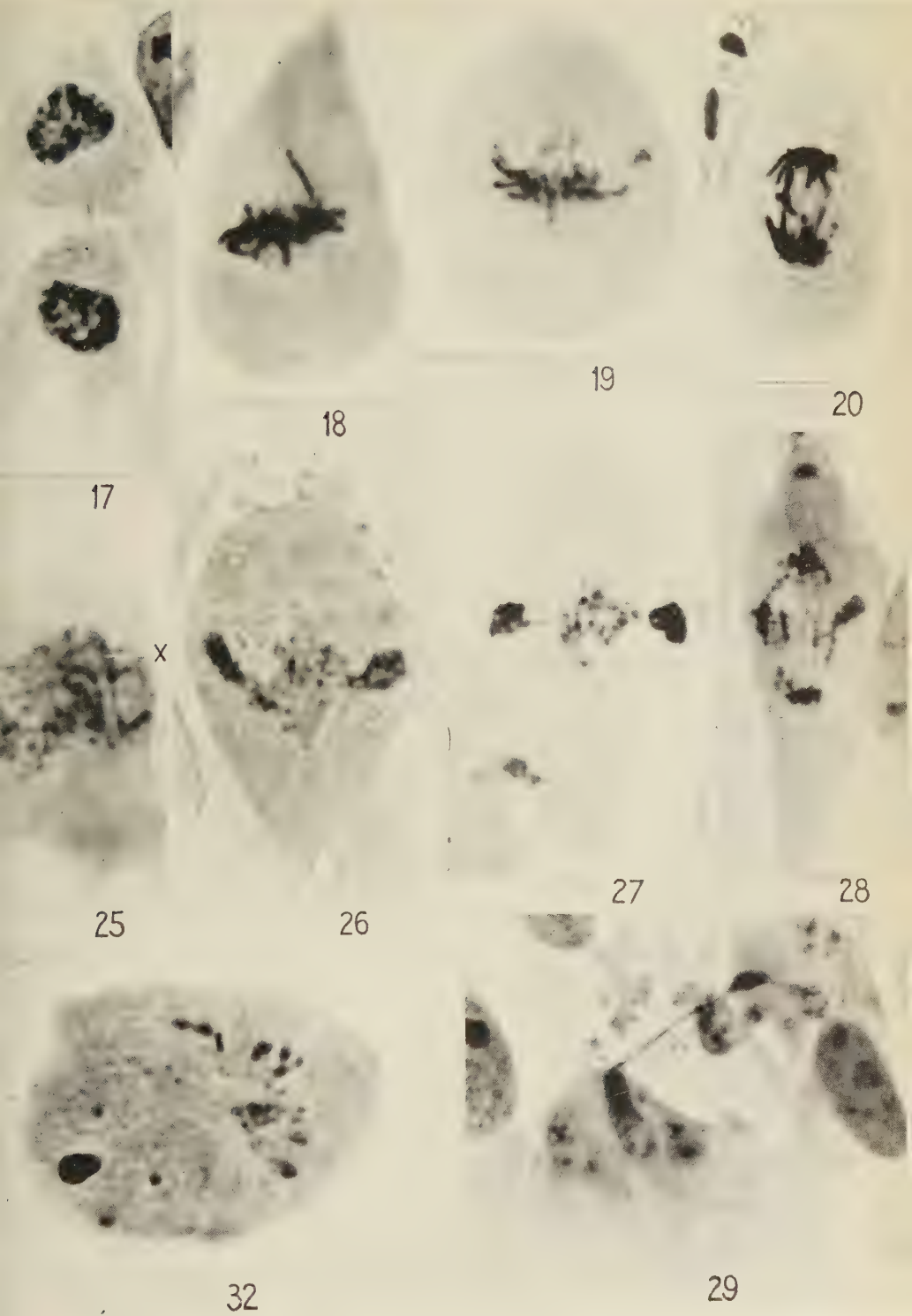


9









A Dye Phosphate for the Histo- and Cytochemical Demonstration of Alkaline Phosphatase, with some Observations on the Differential Behaviour of Nuclear and Extranuclear Enzymes

BY

A. LOVELESS AND J. F. DANIELLI

(From the Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W. 3)

With one Plate

INTRODUCTION

THE method most generally employed for the cytochemical localization of alkaline phosphatase is that due to Takamatsu (1939) and Gomori (1939). It depends upon the hydrolysis by the enzyme of β -glycerophosphate in the presence of calcium nitrate. Calcium phosphate is thus precipitated at the site of enzymic activity, and may be rendered visible by various procedures. The method is unsuitable for tissues containing preformed calcium phosphate, unless they be previously decalcified: furthermore, it involves a number of stages, some of which necessitate a highly critical timing (Danielli, 1946). Lastly, the method as at present employed is not readily adaptable to quantitative histochemical estimations. Another technique, developed mainly by Menten, Junge, and Green (1944), depends upon enzymic hydrolysis of a phenol phosphate, in the presence of a diazonium hydroxide: immediately upon liberation, the free phenol couples with diazonium hydroxide and precipitates *in situ* as an insoluble dye. This procedure is independent of the phosphate residue, and preformed calcium phosphate does not interfere. Nevertheless, the method involves a number of disadvantages, viz.:

- (a) Solutions of diazonium hydroxides are unstable and must be freshly prepared.
- (b) The temperature of incubation must be kept very low, thus denying the enzyme its optimal conditions.
- (c) Diazonium hydroxides exert an inhibitory effect upon the enzyme.

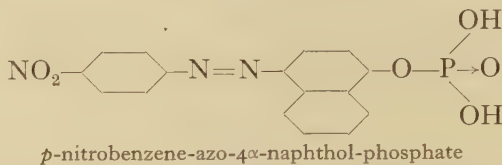
It would clearly be advantageous if a technique were available in which a highly coloured substance is released from a phosphate ester by the action of phosphatase. The properties required of the substance to be released from the ester, ideally, are as follows:

1. It shall be highly coloured.
2. It shall be highly insoluble.
3. It shall not tend to crystallize in the tissue, but remain amorphous: at least, the crystals shall be submicroscopic.

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4. The ester itself shall be at least moderately soluble.
5. The ester shall be stable in the absence of the enzyme.
6. Neither ester nor hydrolysis-products shall be highly inhibitory to the enzyme.
7. The rate of enzyme action on the ester shall be high.

Some of these requirements are not readily compatible with one another, but from work reported previously (Danielli, 1946) it seemed probable that *p*-nitrobenzene-azo-4 α -naphthol-phosphate would comply with many of these conditions.



The phenolic component is, of course, highly coloured. Owing to the presence of a large non-polar moiety and a small polar moiety, it is also highly insoluble in water and alcohol. The ester was expected to be moderately soluble, since the phosphoric acid grouping makes one end of the molecule highly polar and it is known that molecules with a high degree of polar-nonpolar dissymmetry are usually fairly soluble. The reason for this is that the substances form colloidal solutions with the non-polar residues of the molecules lying in the interior of micelles and polar groups at the micelle-water interface. Such solutions should thus contain a low concentration of single molecules of the ester in equilibrium with molecules in micelles. It is doubtful whether an enzyme could act upon the latter, but their presence ensures the maintenance of a supply of single molecules throughout the course of enzyme action.

The chief difficulty anticipated with this ester was that a solution would have detergent properties which might prevent the precipitation of the phenolic component liberated by enzyme action.

PART I. PREPARATION OF *p*-NITROBENZENE-AZO-4 α -NAPHTHOL-PHOSPHATE

Starting from *p*-nitraniline, the ester was synthesized as follows:

1. Diazotization by the 'indirect method' of Saunders (1936), viz. by pouring an aqueous paste of *p*-nitraniline and its equivalent of sodium nitrite on to concentrated hydrochloric acid and ice. The solution was filtered after a few minutes' stirring.
2. Coupling with α -naphthol by stirring the equivalent of α -naphthol in caustic soda solution into the filtered diazotate, with cooling. The *p*-nitrobenzene-azo-4 α -naphthol precipitate was washed with water, alcohol, and ether, and dried, but not recrystallized. (It is recrystallizable with difficulty owing to low solubility in the usual solvents.)
3. Phosphorylation by the method of King and Nicholson (1939), viz. treatment with a slight excess of phosphorus oxychloride in dry pyridine.

Phosphorylation was allowed to proceed for an hour with periodic shaking and the resulting phosphoryl compound decomposed with water.

Hereafter two methods were adopted to obtain a solution of the sodium salt of the phosphoric ester. The solutions obtained by the two methods showed different properties as substrates, as is indicated later.

First solution

The phosphorylated product was diluted with five times its volume of water, throwing most of the unchanged phenol (the phosphorylation does not go to completion) and a part of the ester out of solution. The precipitate was filtered and washed with water to remove pyridine and phosphoric acid. After drying it was digested overnight at 60° C. with 0.4 per cent. sodium veronal adjusted to pH 9.3 with 5N caustic soda. The material was extracted with successive portions of sodium veronal in this way until nothing further was dissolved (as indicated by colour), and the extracts combined and filtered.

The solution, hereinafter referred to as *acid preparation*, had a moderately intense orange colour and showed two components on paper chromatography, viz. an orange band corresponding to the ester and a blue band corresponding to the alkaline (soluble) form of the unchanged phenol. The yield was not good and this may be attributed to two factors: (1) loss of ester during washing of precipitate by solution, (2) rapid acid hydrolysis of the ester immediately upon addition of water after phosphorylation, owing to liberation of phosphoric acid.

Second solution

Saturated baryta was added to the solution after phosphorylation until the solution became strongly alkaline. The precipitated barium salt of the ester was washed exhaustively with water to remove phenol, until the filtrate was no longer blue. It was then washed with 10 per cent. acetic acid to remove barium phosphate. After a final wash with water the barium salt was dried, and an aliquot digested with its equivalent of sodium sulphate in 0.4 per cent. sodium veronal at 60° C. The barium salt of the ester being more soluble than barium sulphate at 60° C., the latter was precipitated and the sodium salt went into solution. The sodium salt itself proved not very soluble and it was necessary to extract the solid material repeatedly with 0.4 per cent. sodium veronal before all was brought into solution. The material thus obtained is referred to hereinafter as *barium preparation*: it had a deep-red coloration, revealed only an orange component on chromatography, and showed a marked detergent activity.

Attempts to obtain the pure dry sodium salt were abandoned in face of three difficulties: (a) low solubility of salt and free ester in the commoner solvents, (b) difficulty of concentrating solutions because of foaming, and (c) tendency of the ester to hydrolyse on prolonged heating of solutions. The concentrations of the solutions employed in these experiments are therefore

defined by preparation and the following table gives quantities employed and yields obtained:

*Quantities of Reagents employed in the Several Stages of Preparation of
p-nitrobenzene-azo-4 α -naphthol-phosphate
Stages 1-3*

<i>p</i> -nitraniline	18 gm.	} in 35 ml. water
Sodium nitrite	10 gm.	
Ice	40 gm.	
Hydrochloric acid	40 ml. conc.	
α -naphthol	23 gm.	} in 400 ml. water
Caustic soda	9 gm.	
Yield of <i>p</i> -nitrobenzene-azo-4 α -naphthol	39 gm. (theoretical 44)	

	<i>Acid preparation</i>	<i>Barium preparation</i>
The phenol	5 gm.	5 gm.
Pyridine	15 ml.	15 ml.
Phosphorous oxychloride	2 ml.	2 ml.
Water	100 ml.	2 ml.
Baryta (Ba(OH) ₂ .8H ₂ O)	..	9 gm. in water
Yield of dry precipitate	5.5 gm.	8.7 gm.
'Free ester' (+phenol)	1 gm.	..
Dry Ba salt	..	2 gm.
0.4 per cent. sodium veronal	500 ml.	2 litres

The yield of solution given in the barium preparation was double that obtained in the 'acid' preparation, and as judged by colour, about four times as strong. However, even supposing a 100 per cent. conversion to the sodium salt, the final solution was less than 0.1 per cent.

PART II. STUDIES WITH RAT KIDNEY

Preparation of Material

Rat kidney was fixed in thin slices with 80 per cent. alcohol, dehydrated in the usual way, and passed to 54° m.p. wax *viâ* methyl benzoate and benzene. Sections were cut at 10 μ and dried on to non-albuminized slides *in vacuo*. After removal of wax, sections were coated with a thin film of celloidin, by passing through a 0.1 per cent. solution before bringing down to water.

Incubation with Substrate

Sections were incubated at 37° C. in the solutions prepared as above to investigate the influence upon enzymic action of the following factors:

- (i) Time of incubation.
- (ii) Degree of dilution of substrate (all dilutions were made with 0.4 per cent. sodium veronal).
- (iii) pH of solution (adjusted by addition of 5N caustic soda or hydrochloric acid).

Results

In these initial experiments no enzyme action was observed using the barium preparation (but see later). When the acid preparation was used, *p*-nitrobenzene-azo-4 α -naphthol was deposited in the brush border and lumina of the proximal convoluted tubules, in most of the nuclei of the tubule epithelium, in nuclei in the glomeruli, and around the adventitia of arterioles—i.e. in all the sites which give a positive reaction with Gomori's technique. The nuclear reaction, however, was far more intense than at other sites and than that shown by Gomori's method, the structure of the nuclei in most cases being completely obliterated by deposits of dye (Pl. I, fig. 1).

Optimal results were given by the strongest solution, 1 hour's incubation being adequate. The substrate was acted upon over a pH range of 7.0–9.5, showing an optimum in the region of 8.7. Variation of pH, time, or dilution did not appear to alter the relative activity of nuclear and extranuclear enzyme. The tissue generally took up unchanged substrate and became yellow stained. This background staining was quickly removed in 50 per cent. alcohol. Most preparations were mounted in glycerine jelly for inspection, but very good mounts were obtained in balsam so long as dehydration and clearing were carried out rapidly. (The dye is slightly soluble in xylene.) Balsam mounts had the advantage of complete absence of background staining.

Further Experiments with the Barium Preparation

Since the enzyme hydrolysed the ester in the 'acid preparation', the question arose as to why it was inactive towards the 'barium preparation'. The only likely impurity in this preparation was sodium sulphate in excess, but this was shown not to inhibit the enzyme when used in Takamatsu and Gomori's incubation mixture. The hypothesis was adopted that, when this substrate is employed, the enzyme cannot act in the total absence of its end-products, since it was known that the acid preparation was contaminated with at least the phenolic component. (It probably also contained phosphate since adequate washing of the phosphorylated product was very difficult to achieve.) A series of solutions was accordingly partially hydrolysed by bringing to pH 2 at 60° for varying times, and kidney sections incubated in these after cooling and readjustment of pH. It was found that only momentary acid treatment was necessary to render the substrate available to the enzyme, but 30 minutes' prehydrolysis was optimal. Brush border activity was very good with these solutions, but nuclear phosphatase gave a far less intense reaction than with the acid preparation. The degree to which hydrolysis had proceeded was checked roughly by paper chromatography of an alkaline solution, and the general indication was that, within limits, the nuclear reaction was more marked the farther preliminary hydrolysis had proceeded (Pl. I, figs. 2 and 3).

Prolonged incubation, i.e. for more than 12 hours, in a non-prehydrolysed solution, gave a heavy deposit of dye in the tubules but no visible nuclear reaction (Pl. I, fig. 4).

From these experiments it seemed probable that the hydrolysis products of the ester were in fact activators of the enzyme. To confirm this, sections were incubated in solutions to which had been added phosphate or the phenol. It was found that as little as 0.1 mg./100 ml. of disodium phosphate was sufficient to instigate enzymic hydrolysis. The picture given was similar to that by Gomori's method, i.e. good brush border deposits but only a moderate nuclear reaction (Pl. I, fig. 5). It was found difficult to bring the phenolic component into solution at pH 9 without prolonged warming which would undoubtedly cause partial hydrolysis of the ester. But sections incubated in an 'inactive' solution to which had been added the solid phenol gave, after 2 hours, a strong reaction in many nuclei with no corresponding action in the brush borders. Staining was, however, confined to nuclei in the innermost region of the cortex, which are normally the most reactive (Pl. I, fig. 6).

Lastly, sections were incubated in (a) a solution which had been completely prehydrolysed, (b) an 'inactive' solution, and (c) a mixture of these: only in the third series was a histochemical reaction shown.

Sections which had been immersed in water at 95° C. for 5 minutes gave no reaction with any solution.

Discussion

It seems fairly clear that, *with this substrate*, end-products of the enzymic hydrolysis must be present at least in trace quantities before enzyme action can start. In this connexion it may be recalled that phosphorylases cannot synthesize glycogen from glucose 1-phosphate unless a trace of glycogen be present *ab initio*. This difficulty can be overcome either by preparing the substrate as for the 'acid preparation' above, or by slight prehydrolysis or addition of phosphate to the 'barium preparation'. One of the latter is advised since the preparative procedure is more straightforward and better yields are obtained.

A differential action is shown by nuclear and extra-nuclear enzymes according to the relative amounts of the two end-products, phenolic or phosphate, present. Thus deposition of dye in the brush border appears to be independent of the prior presence of the phenolic component, but is enhanced by phosphate, whereas nuclear activity is enhanced by the presence of the phenol.

PART III. STUDIES WITH CALCIFYING TISSUES

Since one of the main advantages anticipated from this technique, compared with that of Takamatsu and Gomori, was that the presence of preformed insoluble phosphate should not interfere, some tests have been carried out with dogfish and rat material showing calcifying cartilage and bone. This material, kindly supplied by Miss I. J. Lorch, consisted of 8 μ sections prepared in the same manner as the kidney used above, and cut from the following regions:

1. Transverse sections of the branchial region of dogfish embryo.

2. Transverse sections of the orbital region of dogfish embryo.
3. Longitudinal sections of ribs of the adult rat, passing through the chondrocostal junction.

Sections of rat ribs gave a demonstration of phosphatase activity similar to the Gomori method after 2 hours' incubation at pH 8.7; i.e. a good deposit of dye appeared in the inner layer of the periosteum, in the bone-marrow, and at the chondrocostal junction. Dogfish material did not show a great activity by either method. Although tested at several pH values and at 26° or 37° C., our substrate did not give as good a histochemical picture as obtained by Gomori's technique. Enzyme activity was revealed at the following sites in the times of incubation indicated:

- A. Sections in branchial region: between neural arch cartilages: 15 hours.
- B. Orbital region:
 1. Beneath young dermal denticles, especially buccal teeth: 2 hrs.
 2. Perichondrium of Meckel's cartilage and mandibular arch: 15 hrs.
 3. Outer edge of choroid layer of eye: 2 hrs.
 4. Along pia mater: 2 hrs.
 5. In cavity of mid-brain: 2 hrs.

In no case was any nuclear reaction shown even when solutions optimal for nuclear reaction in kidney were used. The Gomori method gave stronger reactions throughout and also showed up other sites of activity, e.g. around sensory mucous canals, not shown by our method. The technique cannot, therefore, be considered suitable for dogfish material except to reveal sites of comparatively great enzyme activity.

The revelation of phosphatase on the choroid layer is interesting, since it is difficult to observe by Takamatsu and Gomori's method owing to heavy black pigmentation already present. Also phosphatase associated with the denticles is difficult to observe by their method since calcium phosphate already present is not removed satisfactorily by decalcification procedures and a 'blank' staining is therefore produced.

A further very useful feature of this substrate revealed by these tests is that it stains calcified regions strongly yellow within 2 hours, presumably owing to formation of the calcium salt of the unchanged ester; this colour is not removed in alcohol or xylene. Thus a picture is obtained in one operation showing calcified regions (stained yellow) and regions of enzyme activity (stained red). Should any doubt arise as to whether the colour is in fact due to the free phenolic dye and not to 'background' or calcium staining, the doubt may be immediately resolved by testing with dilute caustic soda: this causes the phenol to change to deep blue, but is without effect on the ester. This test cannot be carried out on a section which is to be kept, as the blue alkaline form of the phenol rapidly diffuses out of the tissue.

CONCLUSION

The phosphate ester prepared in this work has most of the properties specified in the introduction. The colour intensity is satisfactory. Some attempts were made to intensify it further, by coupling the phenol liberated by phosphatase with diazonium hydroxides, without success: the degree of alkalinity required for the coupling of further diazo residues brings about rapid dissolution of the dye. The solubility of the dye is very low in acid and neutral solution but increases with alkalinity: up to pH 9, however, it is sufficiently insoluble for the purpose required. It is slightly soluble in alcohol and xylene, so that sections containing it must be passed rapidly through these substances if balsam mounts are required. The introduction of a polar group into the molecule, whilst decreasing its solubility in non-polar solvents, would increase the solubility in water and was therefore not deemed advisable. The dye concerned seems to offer a good compromise between solubility in the two types of solvent. Some difficulty has been experienced with recrystallization in the section as a result of prolonged incubation. When this occurs very long wavy needles are formed which might be mistaken for connective tissue fibres or cell processes. Recrystallization is avoided if incubation is not allowed to proceed for more than 3 hours at 37°.

The ester is not very soluble but sufficiently so. Stronger solutions than those used in this work (approx. 0.1 per cent.) would be of no value, however, owing to high detergent power. On the other hand, the failure of phosphatase in certain sites to act visibly on this substrate might be associated with the diluteness of the solution, since elsewhere, e.g. in kidney, the concentration was found to have a marked effect.

The ester is not completely stable in the absence of the enzyme, becoming increasingly rapidly hydrolysed with decrease of pH. No difficulty should be encountered from this source if the procedure as outlined is adopted and solutions are filtered immediately before use. There is some difficulty about point 7, viz. the rate of hydrolysis of the ester. Whilst some of the sites shown to have activity by Takamatsu and Gomori's method show high activity with the present technique, other sites showing only low activity by their method show none with the new ester. Whilst many factors may in fact be involved, the simplest explanation at present available is that there are many alkaline phosphatases, and a given substrate may be rapidly acted upon by some of these, slowly by others, and by some not at all. If this is so, the ratio of activity towards β -glycerophosphate and *p*-nitrobenzene - azo - 4 α - naphthol - phosphate is not the same for all alkaline phosphatases.

Our opinion is that for general use the new substrate is not as satisfactory as β -glycerophosphate. The sharpness of cytochemical localization is not as great as that obtained with glycerophosphate, and often sites of low activity for β -glycerophosphate appear to have no activity with the new substrate—though occasionally this relationship is reversed. The new method is particu-

larly valuable for pigmented tissues, and for tissues containing preformed calcium phosphate.

In our studies with this new reagent, it was observed with tissue cultures that if a site did not display some phosphatase activity after 4 hours, it would not display activity after even 24 hours. Similar behaviour has been found with the technique of Takamatsu and Gomori, except that the critical period is of the order of 12 hours. With longer incubation all that happens, with both techniques, is an increase in the intensity of reaction in sites which have already displayed activity. We believe this phenomenon to be caused by a physico-chemical limitation which must be common to all techniques involving precipitation of a reaction product, the principle of which is as follows: the formation of a precipitate is dependent upon a threshold number of molecules becoming present simultaneously in a very small unit of volume. Unless this minimum or threshold is reached, precipitation will not occur, even though the solution is theoretically saturated, and the molecules concerned will diffuse until a suitable crystallization centre is reached. Thus a site of enzyme activity can only be revealed if the enzyme activity is sufficient for the threshold condition to be reached—sites of lower activity will always appear negative. Consequently with this type of technique negative results after very prolonged incubation periods do not necessarily indicate complete absence of enzyme, but possibly only that the enzyme activity is below threshold for the method in question.

This investigation has been supported through grants made to the Royal Cancer Hospital by the British Empire Cancer Campaign, the Anna Fuller Fund, and the Jane Coffin Child Memorial Fund for Medical Research.

SUMMARY

1. A new method is described for the cyto- and histochemical demonstration of alkaline phosphatase (monoesterase), a synthetic substrate *p*-nitrobenzene-azo-4 α -naphthol-phosphate being employed as the sodium salt. Two methods for preparing a solution of this substance are described, and optimal conditions for enzymic hydrolysis of the ester worked out by the use of kidney sections.

2. Experiments are also described in which the substrate has been used to demonstrate phosphatase activity in calcifying tissues of dogfish and rat.

3. The results of the experiments with rat kidney indicate that traces of end-products are necessary before the enzyme can attack this substrate.

4. When results obtained with the new substrate are compared with those obtained with β -glycerophosphate, it is found that some sites display more activity towards one substrate than to the other.

5. The cytochemical localization of phosphatase with the new substrate is not as precise as with β -glycerophosphate.

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EXPLANATION OF PLATE I

FIG. 1. Rat kidney incubated 1 hr. in undiluted 'acid preparation' showing strong brush border and nuclear deposit.

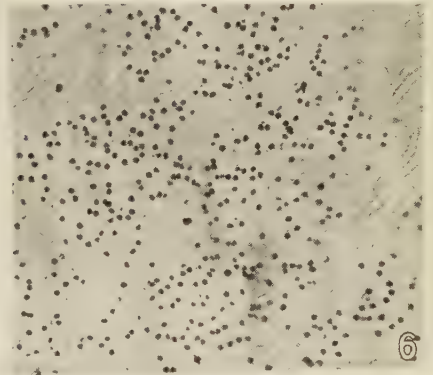
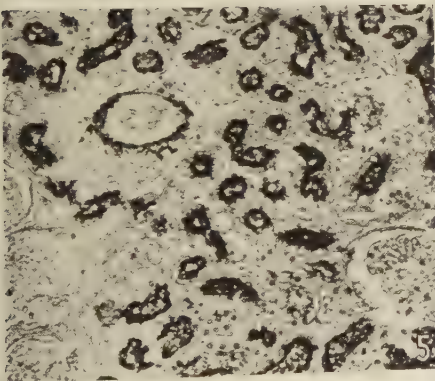
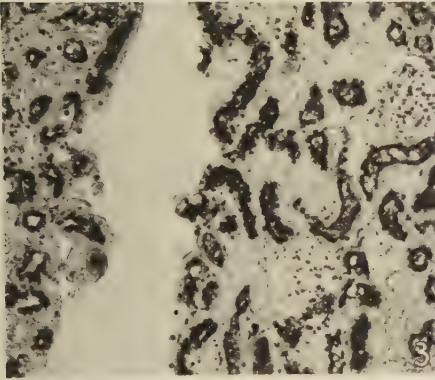
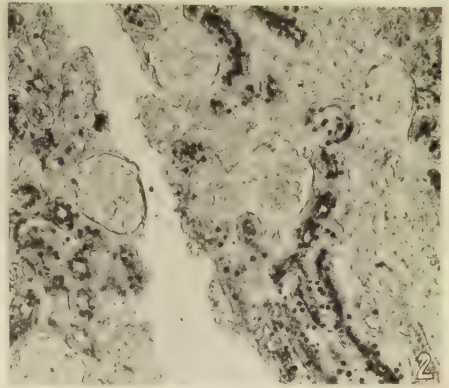
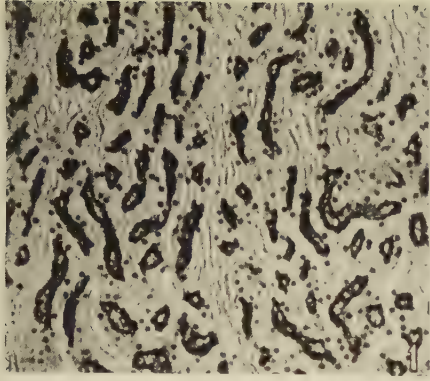
FIG. 2. Rat kidney incubated 1 hr. in half-strength 'barium preparation' after momentary prehydrolysis at pH 2.

FIG. 3. Rat kidney incubated 1 hr. in half-strength 'barium preparation' after 30 minutes' prehydrolysis.

FIG. 4. Rat kidney incubated 15 hrs. in undiluted 'barium preparation' without hydrolysis or addition of end products.

FIG. 5. Rat kidney incubated 1 hr. in half-strength 'barium preparation' containing 1 mg. disodium phosphate /100 ml.

FIG. 6. Rat kidney incubated 2 hrs. in half-strength 'barium preparation' containing added *p*-nitrobenzene-azo-4 α -naphthol.



A. LOVELESS AND J. F. DANIELLI.—PLATE I

A Critical Study of Techniques for the Cytochemical Demonstration of Aldehydes

BY

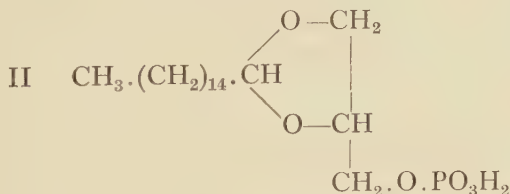
J. F. DANIELLI

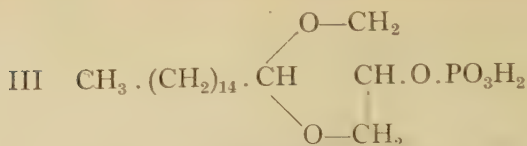
(From the Chester Beatty Research Institute, The Royal Cancer Hospital, London, S.W. 3.)

I. INTRODUCTION

ALDEHYDES, as was shown by Feulgen and Voit (1924), can in principle be demonstrated by treatment with reduced fuchsin. The aldehyde and the reduced dye combine, as in the familiar Feulgen reaction, to give a coloured product. It is clear, however, that if the aldehyde, or the product of its reaction with reduced fuchsin, is diffusible, the results obtained would be worthless from a cytological point of view. Aldehydes can be profitably studied with the aid of existing techniques only if (a) the aldehydes are themselves indiffusible, and (b) if their reaction products with reduced fuchsin are indiffusible. Aldehyde groups having these properties may form part of large colloidal molecules such as proteins and nucleic acids, and also occur as long-chain aliphatic aldehydes such as palmital (see I, below) and stearal (corresponding to palmitic and stearic acids). Aldehydes belonging to the protein-nucleic acid group may be indiffusible in both aqueous and organic solvents. The long-chain aliphatic aldehydes are indiffusible in aqueous media, but diffusible in organic media. Consequently the aliphatic aldehydes, which have been the main group of compounds studied in this work, are the more difficult group to investigate.

The aliphatic aldehydes are only partially present in tissues in the free state. Feulgen and Bersin (1939) have shown that the remaining part is present in an unstable complex, from which the aldehyde may be liberated by brief treatment with cold N/10 hydrochloric acid or with saturated mercuric chloride solution. These authors have also presented strong evidence that the complexes are the acetals of the aldehydes formed with glycerophosphate, with structures II and III.





It is well known that aldehydes are readily liberated from acetals by dilute acid, but it is not altogether clear why mercuric chloride should also bring about this transformation. Furthermore, there is a possibility that mercuric chloride oxidizes compounds containing groups such as $-\text{CO} \cdot \text{CHOH}-$ and $-\text{CO} \cdot \text{CHNH}_2-$ to aldehydes, so that mercuric chloride must be used with discrimination. In Section 7 of this paper, a procedure is given which distinguishes between (a) free aldehyde groups, (b) aldehyde groups bound as acetals, and (c) aldehyde groups liberated by oxidation.

The investigation reported here was made on mouse and rat liver. It was planned to secure answers to the following four questions:

- (i) Is the aldehyde in a fixed preparation in its normal physiological position?
- (ii) Is the method specific for aldehydes?
- (iii) How much aldehyde is destroyed by the procedure?
- (iv) Does the colour developed by the technique appear at the site of aldehyde in the fixed preparation, or at sites having a high affinity for the coloured reaction product?

2. NORMAL PROCEDURE

The procedure for demonstrating aldehydes by the technique of Feulgen and Bersin has been found to give optimal results when used in the following manner:

- (a) Pieces of tissue not more than 2 mm. thick are fixed in a solution containing 8 per cent. formaldehyde and 5 per cent. acetic acid, or 8 per cent. neutral formaldehyde.
- (b) After material has been fixed for not less than 2 hours and not more than 5 days, sections are cut on a freezing microtome.
- (c) The sections are washed with distilled water and then allowed to stand in saturated mercuric chloride solution or cold N. 10 hydrochloric acid for 15 minutes.
- (d) The mercuric chloride or hydrochloric acid solution is sucked off and the sections are washed twice with distilled water.
- (e) Sections are allowed to stand in reduced fuchsin solution for 15 minutes.
- (f) Fuchsin solution is sucked off and the sections washed with a solution containing SO_2 . Three washes each of 5 minutes duration are used. SO_2 solution is made by mixing 10 ml. of 10 per cent. sodium bisulphite solution with 10 ml. of normal hydrochloric acid, and diluting to 200 ml.
- (g) Sections are washed with distilled water and mounted either in neutral balsam or glycerine jelly.

Where sections are robust, they may be shaken during the washings on a mechanical shaker. Otherwise an occasional agitation by hand is necessary to secure adequate penetration of the sections by the various reagents. The purpose of washing with N 10 hydrochloric acid solution is to liberate aldehyde from acetals such as II and III above. Washing with mercuric chloride solution has also been stated to release aldehyde from acetals: in addition it

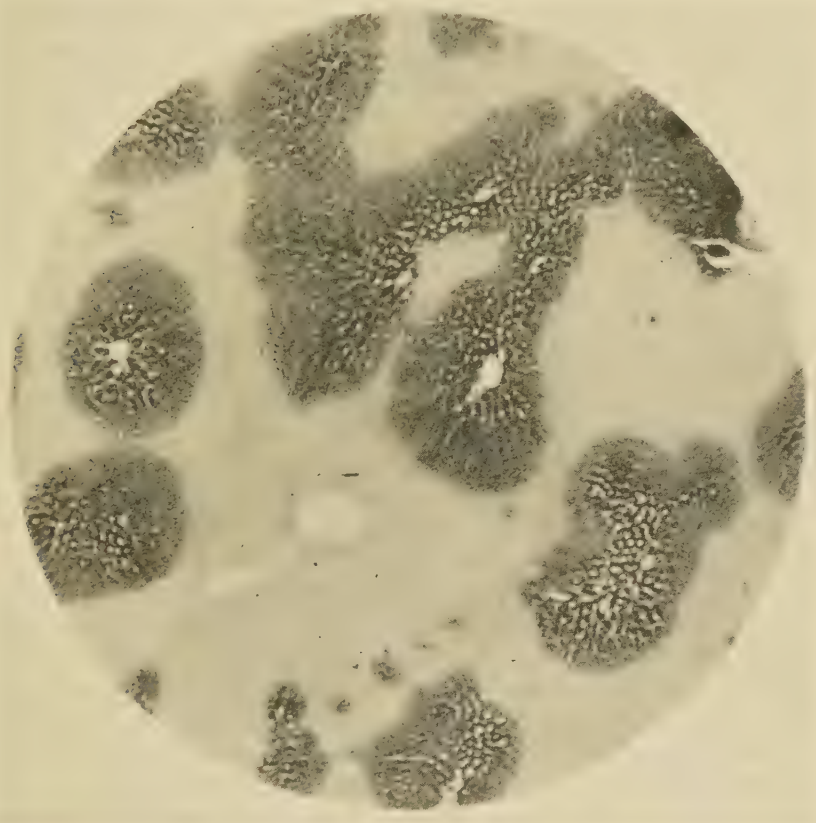


FIG. 1.

may form aldehyde groups by oxidation of compounds containing groups such as $-\text{CO}.\text{CHOH}-$. During step (e) some of the reduced fuchsin usually suffers atmospheric oxidation, and the oxidized dye stains the sections in a non-specific manner. This non-specific stain is removed by the washing with SO_2 solution. Text-fig. 1 shows the characteristic appearance of a rat-liver section treated by this procedure.

3. OTHER FIXATIVES

A considerable variety of fixatives was tried. Some were acid, some neutral, some alkaline, and some contained in addition mercury or formaldehyde.

Furthermore, the effect was tried of pre-precipitation of the material in the tissue by the use of saturated solutions of calcium chloride, magnesium sulphate, or ammonium sulphate. The intensity of the aldehyde reaction, and the nature of its distribution in liver sections, was found to be independent of the physical nature of the fixative. It is therefore clear that the aldehyde revealed by this technique must be occupying its normal physiological position, unless the colour developed appears at a site having a high affinity for the reaction product of reduced fuchsin with aldehyde, and not at the original site of the aldehyde. This latter contingency is an unlikely one, for there is no dependence of the distribution of aldehyde upon the nature of the fixative. Experiments will be described later which clarify the position more precisely.

The fixative components which were tried were formaldehyde, acetic acid, mercuric chloride, pyridine, and trichloroacetic acid. The only mixtures of these substances which were found unsatisfactory were those containing trichloroacetic acid: after fixation in mixtures containing trichloroacetic acid, nuclei frequently stain with reduced fuchsin, without further treatment. It is of course impossible in this technique to use fixatives containing a high concentration of an organic liquid such as alcohol or acetone because the aliphatic aldehydes are soluble in these solvents.

The procedure could be greatly simplified if it were possible to fix tissue in a fixative containing both mercury and reduced fuchsin. Of course formaldehyde could not be used in such a fixative. But so far no success has been obtained with mercury-reduced fuchsin mixtures—possibly because of poor penetration.

The choice of 5 per cent. acetic acid, 8 per cent. formaldehyde as a fixative for general work was based on the facts that the fixation obtained is relatively good, both for the cytoplasm and for the nucleus, and that the formaldehyde present protects the tissue aldehydes from being oxidized by atmospheric oxygen. This fixative was used in all the experiments reported here, unless otherwise indicated. Formaldehyde present in the tissue sections must of course be removed before the section reaches the reduced fuchsin solution. This end is sufficiently procured by the procedure described above. It is not, however, possible to keep material for an unrestricted period of time in this fixative, for polymerization products of formaldehyde are slowly deposited in the fixed material. These polymerization products can also give a colour with reduced fuchsin, which tends to mask the colour developed by the naturally occurring aldehydes. Where it is desired to observe free aldehyde groups only (i.e. not acetal aldehyde), fixation is preferably in 8 per cent. neutral formol.

Some material (e.g. Rous tumour) after fixation in acetic-formaldehyde gives frozen sections which are sticky and difficult to handle. This material can be brought under control by the following procedure: after fixation for at least 2 hours in 5 per cent. acetic acid + 8 per cent. formaldehyde, the pieces are transferred to a Thunberg-tube containing 4 per cent. gelatin at 37° C. to which has been added, just prior to the transfer, 40 per cent. formaldehyde

solution to bring the gelatin up to a content of 4 per cent. formaldehyde. The tube is evacuated and kept at 37° C. for 1 hour, then cooled in ice-water. Frozen sections may be cut as soon as the gelatin has set. No significant destruction of tissue aldehyde is caused by this procedure.

4. TESTS FOR THE SPECIFICITY OF THE REACTION

From the studies of the reactions of aldehydes with reduced fuchsin which have been made by other workers, it seems unlikely that groups other than the aldehyde group CHO can react with reduced fuchsin to give the characteristic purple colour. But to guard against the possibility of other substances being involved, a number of supplementary techniques have been developed.

(a) That the substance involved is fatty in character can be shown by extracting sections with fat solvents between steps (b) and (e) of the procedure given in Section 2 of this paper. Washing may be carried out with alcohol-ether mixtures followed by xylene, or by acetone only. The extraction should be carried out for at least 15 minutes, during which three changes are made of the extracting fluid. The aldehyde is almost entirely removed by extraction.

(b) A characteristic reaction of aldehydes is the development of a purple colour with azobenzene phenylhydrazine sulphonic acid. The procedure is as follows: sections are taken through the procedure given in Section 2 of this paper up to step (d). Individual sections are then removed from the bath and placed in a solution composed of 7 drops of a saturated aqueous solution of the azobenzene phenylhydrazine sulphonic acid and 4 drops of concentrated sulphuric acid in the bottom of a test-tube. This is heated in a boiling-water bath for 30 seconds, after which a large excess of water is added. The sections are then mounted on a slide in concentrated hydrochloric acid. Under these conditions the aldehydes form a compound with azobenzene phenylhydrazine which is purple. The distribution of the purple colour so found is similar to the distribution of the colour found with Feulgen's method. Although the distribution of the colour is the same as that found in Feulgen's method, the technique is not suitable for general use because fairly rapid hydrolysis of the tissue takes place in the strong acid which is used as a mounting medium. If not mounted in strong acid, the aldehyde is coloured a pale yellow only, which is unsatisfactory for general purposes.

(c) A characteristic reaction of aldehydes is their ability to reduce ammoniacal silver nitrate, giving a black precipitate. The silver solution is prepared by adding concentrated ammonia solution to 5 per cent. silver nitrate solution in water, until the precipitate which forms at first is completely redissolved. Sections are placed in this solution and left for 2 hours at 60° C. in the dark. The distribution of the black precipitate so obtained in liver sections, taken after step (d) of the procedure given in Section 2 of this paper, is similar to the distribution of colour obtained with the reduced fuchsin method.

(d) A characteristic reaction of aldehydes is the formation of a yellow hydrazone with 2:4 dinitrophenylhydrazine. A saturated solution of 2:4 dinitrophenylhydrazine in N hydrochloric acid is cooled to 0° C. Sections

from step 2(*d*) are placed in this solution and left for either 2 hours or 20 hours. They are then washed quickly in cold N hydrochloric acid and mounted in glycerine jelly. In liver sections the distribution of colour so obtained is similar to that obtained with the reduced fuchsin method.

(*e*) A characteristic reaction of aldehydes is their ability to form oximes by combination with hydroxylamine. If, therefore, the purple colour formed with reduced fuchsin is due to combination of the reduced fuchsin with aldehyde groups, then prior treatment with hydroxylamine should eliminate the purple colour. The procedure is as follows:

2 gm. of solid hydroxylamine hydrochloride and 4 gm. of sodium acetate are dissolved in 6 ml. of distilled water. Sections are taken through the procedure given in Section 2 of this paper for steps (*a*) to (*d*) inclusive, and then are placed in the hydroxylamine solution at room temperature, and left for 1 hour. The sections are washed in distilled water and treated with the reduced fuchsin solution, &c., as in steps (*e*) to (*g*) of the procedure in Section 2. When liver sections are treated in this way, the appearance of colour with reduced fuchsin is entirely eliminated.

These five supplementary tests prove conclusively that the colour obtained with reduced fuchsin in liver sections is due to the presence in the sections of aldehydes. The solubility of the aldehydes in lipid solvents shows that these aldehydes are fatty in character. These tests may of course be applied to practically any other tissue.

5. THE DEGREE OF DESTRUCTION OF ALDEHYDE

The possibility exists that aldehyde is partly destroyed by the procedure. This has been tested by increasing the duration of each step in turn in the routine by a factor of fourfold. This can be done without causing any significant loss of the colour formed by reduced fuchsin in the case of liver sections. It is therefore clear that no substantial loss of aldehyde is caused by any of the steps in the procedure. It is, however, necessary that step 2(*a*) should not occupy more than 5 days for the best results. Keeping tissue for longer periods than this in the formaldehyde-acetic mixture may result in the deposition of other aldehydic material in the tissue; and if the tissue is removed from formaldehyde, the naturally occurring aldehydes in the tissue tend to be lost by oxidation.

6. THE RELATIONSHIP BETWEEN THE SITE OF ALDEHYDES AND THE POSITION OF THE COLOUR IN THE FUCHSIN REACTION

The possibility exists that the purple colour obtained with the reduced fuchsin reagent diffuses to regions which have a high affinity for this coloured substance. If this is so, the results obtained will not show the original site of the aldehyde. It has been pointed out, in Section 3, that this is an unlikely contingency. It is made still more unlikely by the fact that the distribution of colours in Tests 4(*b*), (*c*), and (*d*) is the same as that of the fuchsin. Furthermore, the localization of the purple colour may be very sharp. Occasionally in

the liver one intensely stained cell will be seen, completely surrounded by quite unstained cells, and in this cell the greater part of the aldehyde may be sharply localized in one part of the cytoplasm. The nucleus is practically always unstained.

However, the position can be made more secure by getting the aldehydes into a diffusible condition, combining them with reduced fuchsin, and then placing sections from which all aldehyde has been removed in this mixture. The positions in which colour is taken up will be those which have a high affinity for the aldehyde-fuchsin compound. The procedure used for this was to extract rat-liver with alcohol and then with xylene, in an atmosphere of nitrogen. The alcohol and xylene were boiled off *in vacuo* and the residue dissolved in a small volume of alcohol. When this alcoholic solution was poured into water it gave a colloidal solution. To this was added reduced fuchsin, whereupon a purple colour appeared almost immediately. Into this solution were placed sections from which all the aldehyde had been removed by extraction, as in procedure 4(a). After 15 minutes the sections were removed and washed in SO_2 solution. It was found that the nuclei were intensely stained and there was a uniform diffuse staining of the cytoplasm. There was no highly localized staining of the cytoplasm. This distribution of colour is quite different from that found with the procedure given in Section 2.

One can therefore conclude that the purple colour does in fact appear at the site of aldehyde occurrence. If the aldehyde were able to diffuse, a quite different cytological picture would be obtained from that given by the procedure given in Section 2.

7. DIFFERENTIATION BETWEEN FREE ALDEHYDE, ALDEHYDE ACETAL, AND ALDEHYDE LIBERATED BY OXIDATION

According to Wislocki and Dempsey, aldehydes may be formed from α -hydroxy ketones by cold aqueous mercuric chloride. Boscott, Mandl, Danielli, and Shoppee (1948) have demonstrated that this is not so with typical α -hydroxy ketones, such as deoxycorticosterone. Nevertheless, it is conceivable that atypical compounds would be oxidized to aldehydes under these conditions. Thus it may be necessary at times to discriminate between (i) free aldehydes, (ii) aldehydes present as acetals, and (iii) aldehydes liberated by oxidation. This can be done as follows, after fixation with neutral formaldehyde solution:

- (i) Expose to reduced fuchsin, omitting treatment with mercuric chloride or hydrochloric acid in steps (c) and (d) of the general procedure of Section 2. This demonstrates free aldehyde only. Care must be taken to avoid using an acidic fuchsin solution.
- (ii) Use of N/10 hydrochloric acid in steps (c) and (d) will show both free and acetal aldehyde. But if the free aldehyde is converted to oxime, as in procedure 5(e), before steps (c) and (d), only acetal aldehyde will be demonstrated.
- (iii) Use of mercuric chloride solution in steps (c) and (d) may show, in

addition to free aldehyde and acetal aldehyde, aldehyde formed by oxidation. Treatment with cold N/10 HCl, followed by oxime formation, then by mercury treatment, shows only aldehyde formed by oxidation.

8. DISCUSSION

In the introduction to this paper it was pointed out that satisfactory answers to four questions must be obtained before the reduced fuchsin method for demonstration of aldehydes could be regarded as satisfactory. The experiments described in Sections 2 to 6 of this paper define conditions under which the procedure may be carried out, so as to obtain results which comply with the restrictions and requirements of these four questions. The supplementary procedures 4(b), (c), and (d) are not as satisfactory as the reaction with reduced fuchsin. But they are very necessary supplementary techniques for making certain that the colour developed is in fact due to aldehyde groups. As a routine check that the purple colour formed after treatment with reduced fuchsin is indeed caused by the presence of aldehydes in the material, it is best to use test 4(e). With this test all colour caused by presence of aldehydes is eliminated by formation of the aldoxime before exposure to reduced fuchsin.

The chemical specificity of the reaction with reduced fuchsin for aldehydes has been examined on the test-tube scale by a number of other workers, particularly by Oster and Oster (1946). The work of Oster and Oster shows that none of the other compounds likely to occur in tissue sections will in fact give a purple colour under the conditions they use. The main possible source of error lies in the resemblance between ketones and aldehydes. But the experiments of Oster and Oster seem to make it clear that none of the known ketones will in fact react with reduced fuchsin. There is, however, clearly a need for a reaction which is specific for keto groups.

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SUMMARY

A critical study has been made of Feulgen's technique for demonstrating tissue aldehydes. Conditions are given within which the technique is reliable on the cytological scale. Five supplementary tests for aldehydes are described. It is shown how distinction may be made between free aldehyde, acetal aldehyde, and aldehyde formed by oxidation.

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On the Significance of the Plasmal Reaction

BY

A. J. CAIN

(From the Department of Zoology and Comparative Anatomy, Oxford)

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INTRODUCTION

THE 'plasmal reaction' has been widely used in cytology, and is generally interpreted as revealing the presence of aldehydes, but there is considerable doubt as to their source. According to Feulgen and his collaborators, who first described the reaction, the aldehydes (plasmal) are formed by decomposition of acetalphosphatides (plasmalogen) under the catalytic influence of mercuric chloride. According to Verne, they are formed by oxidation at unsaturated linkages in various forms of lipoid, the explanation of the action of mercuric chloride being either that it 'unmasks' the lipoids (Gérard) or that it destroys the inhibiting effect produced by formaldehyde when that substance is used in fixation.

The techniques used for demonstrating the plasmal reaction vary considerably, having in common only the use of Schiff's reagent and the production of results in the cytoplasm and its inclusions, not in the nucleus.

This paper presents evidence to show that the variations in technique are of importance and affect the results. There is no evidence that aldehydes are concerned in the reaction as practised by cytologists, but there is good evidence that oxides and hydroperoxides are the groups responsible in many cases, and that although acetal linkages will give rise to aldehydes under the conditions of the improved technique suggested, there is no *histochemical* evidence that such linkages are in fact present in the tissues investigated. Such substances have been prepared from tissues by Feulgen and his school, but the plasmal technique as usually practised does not show them clearly.

COMPARISON OF TECHNIQUES

The work of Feulgen and his school has been reviewed from the biochemical standpoint by Feulgen and Bersin (1939) and completed by the synthesis of an acetalphosphatide (Bersin *et al.*, 1941). Imhäuser (1927) carried out a [Quarterly Journal Microscopical Science, Vol. 90, part 1, March 1949]

survey of the occurrence of plasmal in tissues, and concluded that it was very widespread indeed. The technique was described by him and by Feulgen and Voit (1924).

Verne has been the principal investigator of plasmal and has modified the technique. He considers that positive results are due to the production of aldehydes by the atmospheric oxidation of double bonds (1928*a, b, c*, 1929*a, b*, 1936*a, b*, 1937*a, c*). Lison (1932) showed that Schiff's reagent would react with oleic acid and certain other unsaturated substances besides aldehydes. He noticed that not all of these would react with other compounds used to characterize aldehydes, nor did a reaction with one of these compounds mean that the substance would react with others. Oxidant enzymes might also recolour Schiff's reagent. Gérard (1935) believed that, myelin excepted, a positive result was always due to 'oxidases'. Lison (1936*a*) showed that weak positive results with the Nadi reagent were due to peroxides formed in lipoids by atmospheric oxidation. Verne (1937*b*, 1940) disagreed with these conclusions and reaffirmed his theory that aldehydes were responsible.

The techniques used were as follows. All employ sections cut on the freezing microtome.

1. *Technique of the Feulgen School*

Frozen sections of fresh tissue are used, some being treated with mercuric chloride solution, the rest acting as controls. Untreated sections negative or only weakly positive with Schiff's reagent, treated sections intensely positive.

Since this is the technique prescribed by Feulgen, it alone can be called the plasmal technique.

2. *Technique of Verne*

Tissues are fixed in a mercuric chloride or platinum chloride fixative. No control section.

Lison (1933) referred to this as the Feulgen-Verne technique (F.V.), a name usually employed by Verne himself.

3. *Technique of Gérard*

Tissues are fixed in a formaldehyde-fixative which is carefully washed out. A control section is used, which is left in distilled water while its fellow is in mercuric chloride solution.

4. *Technique of Guyon (1932)*

This appears to be the plasmal technique as applied to myelin. Both Guyon and Verne refer to positive results with fresh tissue. This does not, apparently, mean that fresh tissue will recolorize Schiff's reagent. A short treatment with mercuric chloride is necessary. However, Verne (1937*a*, p. 4) does seem to refer to a positive result without mercuric chloride.

Most investigators have used preliminary treatments with phenylhydrazine or semicarbazide or their derivatives to prevent the reaction. Sodium

bisulphite and dimedone have also been employed. But these will react with compounds other than aldehydes, and most are strong reducing agents which might be expected to prevent atmospheric oxidations. Their value in histochemistry is discussed below (p. 82).

EXPERIMENTAL RESULTS

The following questions arise from the preceding discussion:

What is the relation between the results obtained by the various techniques?

How far can the phenomena observed be reproduced with unsaturated lipoids *in vitro*?

Is the technique entitled to rank as a histochemical test?

They will be dealt with in the order given.

A. *The Relations between the Various Techniques proposed*

Feulgen's original technique—the plasmal technique as it will be called in this paper—was tried on mouse testis, which should show a positive result in the interstitial cells and a negative or very weak one elsewhere. Frozen sections of fresh tissue were stuck on to slides coated with albumen by gentle and rapid heating; some were treated with mercuric chloride solution, others passed directly into Schiff's reagent. After 10 minutes the slides were washed carefully in sulphur dioxide water, as prescribed by Verne (1929*a*, repeated by Lison, 1936*b*, p. 216), to remove all traces of Schiff's reagent and so prevent the subsequent reformation of basic fuchsin. Sections were also used which had not been subjected to heat.

This technique is unsuitable for all those tissues that will not retain their coherence on thawing after sectioning. The control sections, both heated and unheated, showed a very faint purplish-red coloration throughout, which was also seen in places where there was an appreciable amount of albumen. Schiff's reagent is very difficult to wash out thoroughly, even from sections, and this general coloration is considered to have no significance. The sections treated with mercuric chloride solution showed a much deeper, patchier, and rather bluer coloration, but were too badly smeared to allow its precise location to be determined.

The Feulgen-Verne technique was tried on the same material, but fixation was in saturated mercuric chloride solution, that concentration being considered more suitable, and was for 6 hours only. Treatment with Schiff's reagent gave a positive result which was more feeble than that obtained by the plasmal technique. Gérard's technique, formal-calcium (Baker, 1944) being used as fixative for 6 hours, gave an extremely feeble, almost negative, result with the control sections, and a positive intermediate between the plasmal and Feulgen-Verne results with the sections treated with mercuric chloride.

Adrenals of mature male and immature male and female white rats were used for a more extended trial of Gérard's method. It was found that after

6 hours' fixation, the medulla was almost negative; the cortex showed a positive result only in the zona glomerulosa and the innermost region of the zona reticularis; these results were exceedingly feeble and the intensification with mercuric chloride was very slight indeed. But sections left overnight in water showed a much more positive result in the cortex, which appeared to follow the distribution of lipoids very closely, being most marked in the outer region of the zona fasciculata where the cells are very heavily laden. The picture seen was exactly comparable with those obtained on the same material with Sudan IV or Sudan black (Harrison and Cain, 1947, pl. I, fig. 2). There was no visible difference between the controls and treated sections. The Feulgen-Verne technique (with fixation for 6 hours) gave the same results except that the sections were brighter throughout, and the medulla was as bright as the cortex. Sections left overnight in water showed a considerable intensification of the colouring in the cortex, and a slight reduction in the medulla.

As frozen sections of fresh tissue were of very little use, observations were made macroscopically on small pieces of tissue. Portions of mouse skin, body-wall, liver, kidney, testis, and adrenal were dropped into Schiff's reagent. Colouring was seen only after 20 minutes, and then principally at the cut surfaces. It spread and intensified slowly over a period of hours. But pieces of the same tissues dropped into Schiff's reagent containing mercuric chloride behaved very differently. An intense reaction was seen throughout the tissue (except in the case of liver and of adipose tissue) within 5 minutes, and it had apparently reached its maximum within 15. The colour produced was a very deep violet. This reaction differs remarkably in speed, colour, and intensity from those previously seen.

The relation between this intense reaction and that obtained on fixed tissues was investigated by repeating the experiment with mouse skin, body-wall, kidney, and liver (to act as control), after various periods of fixation in mercuric chloride and in formal-calcium, and comparing the intensity and shade of the resulting coloration. It was found that after 2 hours' fixation in mercuric chloride solution, the reaction was definitely weaker. After formal-calcium, followed by an hour's washing in running water to remove the formaldehyde, only a feeble reddish-purple coloration was produced. After 2 hours in the reagent (Schiff plus mercuric chloride) both lots of tissue were considerably more intensely coloured, though by no means as dark as the unfixed tissue, and the formaldehyde-fixed material was still somewhat redder, though much less so than at first. After 6 hours' fixation, the mercuric chloride-fixed pieces showed a coloration slightly paler than that obtained after 2 hours; liver was slightly more coloured than before. The formaldehyde-fixed pieces were much weaker. After 24 hours, the reaction was not prevented but was comparatively very weak, and there was a strong tendency for the liver to colour.

It would seem, then, that there are two distinct phenomena involved. On the one hand is the intense and rapid reaction given by fresh tissue treated

with a mixture of Schiff's reagent and mercuric chloride, which is progressively reduced by fixation, and apparently affected specially by formaldehyde. On the other is the reaction seen in adrenal cortex, slower, increasing with time, obviously closely connected with the amount of lipoid present, and affected comparatively slightly by treatment with mercuric chloride. It appears to be this reaction that is shown by the techniques of Feulgen-Verne, and of Gérard, if fixation is prolonged (more than 3 hours), with a trace of the other if treatment with Schiff is carried out on sections not more than 4 or 5 hours after the tissue is placed in the fixative.

As it is so difficult to wash out Schiff's reagent, and previous fixation is not to be recommended for studying the rapid reaction, an observation on the solubilities of Schiff-compounds (further discussed below) was used in an attempt to improve on the plasmal technique. The compound between formaldehyde and Schiff's reagent is water-soluble and, as will be seen from its formula,



it is a very feeble dye. The following technique was therefore employed:

1. Drop pieces of fresh tissue into a mixture of equal parts of
 - (a) Schiff's reagent diluted with its own volume of sulphur-dioxide water, and
 - (b) a saturated aqueous solution of mercuric chloride.

For controls, drop other pieces into the diluted Schiff's reagent, without addition of mercuric chloride.

Allow the pieces to remain in these solutions for 15 minutes.

2. Remove tissues, wash in sulphur-dioxide water to remove the excess Schiff's reagent without allowing recoloration, and drop immediately into formalin (40 per cent. formaldehyde). Leave for 2 hours.

The formaldehyde-Schiff compound forms rapidly, and the control pieces become almost as dark violet as the others.

3. Wash in running water for 2 hours or more until the control pieces have become only a very pale lilac. This washing also removes the formaldehyde before embedding.
4. Either cut sections without embedding, or embed in gelatine overnight, harden in formalin, cut sections, and mount in Farrants's medium. As the colour begins to pale, these operations should be carried out as quickly as possible.

This method is applicable only to very small pieces of tissue, as penetration by Schiff's reagent is poor. The results obtained were good, the stain being very intense and apparently very selective, especially in skin. It was noticeable that fat droplets were completely negative but the cytoplasm of the fat-cells was heavily positive. Sebaceous glands were negative, the bases of the hair

follicles feebly positive, muscle and myelin sheaths very positive. Remarkably enough, small elements scattered in the connective tissue are feebly positive; these appear to be nuclei of connective tissue cells. The nuclei of the fat-cells also appear to be coloured faintly. A control section should therefore be coloured with Sudan black, to demonstrate the lipoids present, and only positive results obtained on lipoids should be considered. In mouse adrenal, the medulla was negative, the cytoplasm of most of the cortical cells positive, the lipid droplets completely negative. The control sections were colourless in all cases.

Sections prepared by the Feulgen-Verne technique showed a much more feeble coloration or none at all.

B. Experiments with Unsaturated Lipoids in vitro

Oleic and linoleic acids were available for these experiments. In general, it was found that, as might be expected, linoleic acid reacted more rapidly and gave more intense results than did oleic acid. Presumably arachidonic acid would be more reactive still. In interpreting the results it must be remembered that pure lipoids are well known to be much more slowly oxidized than impure ones, and that lipoids in tissues may well react far faster than the same substances when extracted.

Both oleic and linoleic acids will react with Schiff's reagent after about an hour, producing a fine purple, which is certainly not the colour of basic fuchsin. It is worth noting that if a thin layer of oleic acid is allowed to lie on the Schiff's reagent in a test-tube without disturbance, the colour is formed at the interface and shows a strong tendency to diffuse in the aqueous fluid, the rest of the oleic acid being uncoloured. If, however, the tube is continually shaken, the oleic acid gradually becomes coloured, and the Schiff's reagent is only slightly tinged. The same effect can be seen with linoleic acid.

Both oleic and linoleic acids if exposed to the air will become coloured by Schiff's reagent within 15 minutes. After prolonged exposure (several days) coloration takes place within 2 or 3 minutes. Linoleic acid solidifies as it oxidizes, oleic acid does not. Strips of filter-paper soaked in the acids and exposed on clock-glasses protected from dust are convenient for these experiments. If they are exposed to mercuric chloride solution, oxidation is very much more rapid. Linoleic acid exposed to the air for 18 hours colours with Schiff's reagent in 10 minutes. If left in contact with mercuric chloride for that length of time, it colours almost instantaneously. After treatment with saturated mercuric chloride solution for 15 minutes the unoxidized acids showed no shortening of the time taken to colour with Schiff's reagent. Partly oxidized acids showed a very slight shortening and intensification of the colour. The extremely high rate of oxidation of the lecithins is well known, and arachidonic acid is particularly associated with them (Hilditch, 1947). It is possible that a pseudoplasmal reaction might be caused through acceleration of the oxidation of highly unsaturated acids by mercuric chloride.

Unoxidized oleic and linoleic acid can be prevented from oxidizing by treatment with phenylhydrazine, 2-4 dinitrophenylhydrazine, and semicarbazide. The oxidized acids recolour Schiff's reagent in the same time as do samples of oxidized acid untreated with these reagents. As neither the pure nor the oxidized acids will reduce ammoniacal silver nitrate even on boiling, it appears that aldehyde groups are not formed during atmospheric oxidation for at least a week, and that phenylhydrazine and similar reagents, being reducers, can block further oxidation by their presence, as do carotenoids dissolved in fats. It was noticed that the acids became darker and redder in colour, indicating that the phenylhydrazine was being oxidized.

In contrast to the above, palmital and stearyl, after treatment with phenylhydrazine, were completely negative to Schiff's reagent, and remained so after immersion in it for 24 hours. The phenylhydrazine was not decomposed by the acidity of the reagent within this period.

Verne (1929*b*) remarks that by treatment with phenylhydrazine for 24 hours, the staining is abolished in both the cortex and medulla of the adrenal. This is confirmed for the adrenal of the rat. Staining in the medulla is discussed below.

C. The Status of the Plasmal Reaction as a Histochemical Test

Danielli (1947) has shown that the product of the reaction between Schiff's reagent and desoxyribosenucleic acid is water-soluble, diffusible, and readily taken up by chromosomes when used as a stain, the rest of the preparation being coloured faintly. The colour produced with oleic and linoleic acids is diffusible, as mentioned above, in the Schiff's reagent. Slices of an oleic acid-gelatine emulsion fixed in formaldehyde and carefully washed were coloured and placed in various solutions. The colour appears to be slightly soluble in very dilute acids, more so in stronger ones, but insoluble in alkaline solutions and in alcohol.

The coloured substance produced from Schiff's reagent and acetaldehyde is very slightly soluble in distilled water, insoluble in alkaline solutions, and very slightly in acid ones, but readily soluble in alcohol. It is a very deep violet. The substance produced from Schiff's reagent and formaldehyde is easily soluble in distilled water and dilute acids and alkalis, but only slightly in alcohol. That produced from palmital and stearyl appears to be insoluble in water, dilute acids, and dilute alkalis, but easily soluble in alcohol. In aqueous suspension it shows no dyeing power.

The properties of the formaldehyde-compound were further investigated. Sections of mouse skin placed in an aqueous solution became violet but lost all their colour on washing, except that the hair-shafts remained very faintly purple for some time. Sections of rat adrenal took up the colour on prolonged immersion, the heavily laden fat-cells in the cortex being coloured most deeply. The medulla was scarcely tinged. Immersions up to an hour produced almost no effect. Also, as mentioned above, the colour even when produced *in situ* can be washed out completely.

It appears, then, that while the lower members of this series may be water-soluble in certain circumstances, the higher ones are not, and none have any appreciable tinctorial power. The slightest contact with regenerated basic fuchsin is far more dangerous than an hour's soaking in a solution of one of these substances; fortunately, the colour produced is a very light red, and stains almost all parts of a section indiscriminately. However, as Lison has shown, colour is not always a trustworthy guide. But if one section in a series is coloured much more red (less blue) than others, it should be regarded with suspicion.

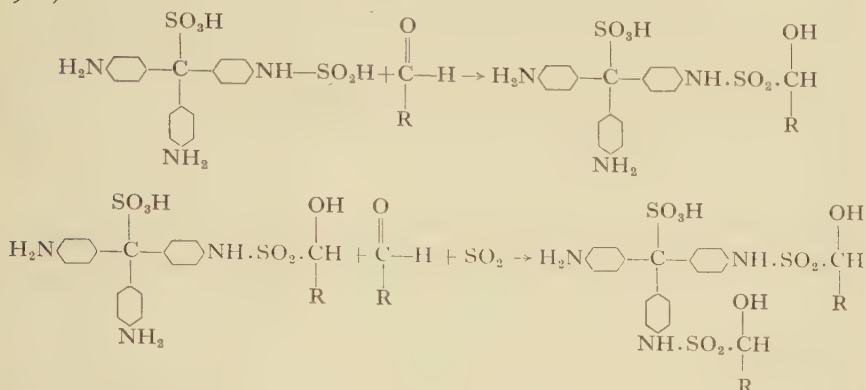
DISCUSSION

It appears that in the past two distinct reactions have been confused under the name of 'plasmal', the intense rapid initial coloration, which is apparently due to 'plasmal' in Feulgen's sense, and the slow reaction, increasing with passage of time and only slightly affected, if at all, by mercuric chloride, which is due to autoxidation products of unsaturated lipoids.

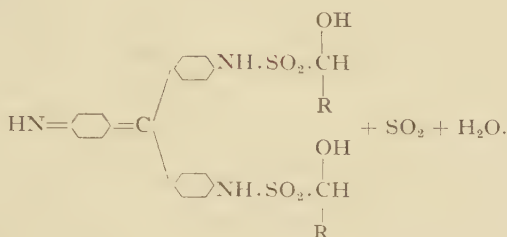
The first, the plasmal reaction, is seen at its most intense when fresh tissue is acted upon by a mixture of Schiff's reagent and mercuric chloride. Schiff's reagent by itself showed no positive result within 15 minutes, or only an exceedingly feeble one, on the tissues examined. This reaction is, as Imhäuser showed, very widespread. Liver is almost negative, but the other tissues examined show intense colorations. Fixation reduces the intensity of the reaction progressively, and it is interesting to note that pieces of tissue give a more intense reaction than do sections cut without embedding from the same pieces before treatment. Palmital and stearal polymerize very readily, and are, in general, very reactive substances. Presumably the increased exposure consequent upon section-cutting assists in their alteration.

Fat globules are totally unaffected by this reaction. In the other, they appear to be the principal sites of it, and stain intensely in the cortex of the adrenal in sections which have been left in water overnight. Verne considers the resulting coloration as a positive result for plasmal. But the distribution of the colour has altered considerably, and the characteristics of the reaction are quite different. It increases in intensity with the passage of time, and mercuric chloride has little or no effect. The work of Gérard and of Lison shows that oxidizers appear in connexion with this reaction in certain cases at least. This is in agreement with part of the chemical evidence on the autoxidation of unsaturated lipoids, during which process many and varied radicals may appear (Markley, 1947; Hilditch, 1947). If it is known that one is dealing with a single substance, or even with two, then possibly they may be identified by the use of a large number of reagents undergoing different types of reaction. In this particular case the number of radicals is unknown, and the reactions available appear to fall into only three groups—reduction, addition, and condensation, as exemplified by ammoniacal silver nitrate, sodium bisulphite, and phenylhydrazine. The reaction of aldehydes with Schiff's reagent is an

addition followed by a condensation, according to Wieland and Scheuing (1920):



which rearranges to:



but as Lison has shown, oleic acid can react, as the elements of water are supplied entirely from the Schiff's reagent. The reaction with silver nitrate, which, as Verne remarks, is not easy to carry out on sections, is also given by ethylene oxides, and addition reactions can also take place across carbon-carbon double bonds; and although phenylhydrazine and similar compounds appear to be specific for the $> \text{C} = \text{O}$ group, their reducing-power can interfere with the reactions of groups other than aldehydes and ketones. Sodium bisulphite and dimedone (Heilbron and Bunbury, 1943) are also reducing agents. In these circumstances no certain conclusion can be drawn as to the composition of reacting mixtures. The only conditions under which any definite statement could be made about reactions in tissues are found when the reaction can be reproduced *in vitro*, and analysis of the extract shows that only one substance is responsible; these conditions have never been satisfied.

In the case of the plasmal reaction, there is one other characteristic, which is of the utmost importance, namely, the speed at which the reaction is produced under the influence of mercuric chloride. It has been shown above that the speed of autoxidation of unsaturated fatty acid radicles can be greatly increased by the presence of mercuric chloride, the normal and the catalysed rates both increasing with the degree of unsaturation. Studies on arachidonic acid are unfortunately not available, and the possibility should be borne in mind that a short treatment with mercuric chloride of fresh tissues containing

it might cause an appreciable intensification with Schiff's reagent. But the speed of breakage of the acetal linkage is so great, as Feulgen showed, that it seems reasonable to treat positive results obtained within 10 minutes on sections or small pieces of tissue as being due to the liberation of plasmal. However, there is no absolute certainty. A prolonged treatment with Schiff's reagent must not be used because the oleic and other unsaturated radicles might begin to react directly.

The evidence that this reaction, produced rapidly under the influence of mercuric chloride, is due to the liberation of palmital, stearyl, and other aldehydes from acetalphosphatides, is entirely the work of Feulgen's school, and its interpretation in that sense rests entirely upon this basis. Consequently, in carrying out the reaction care must be taken that the necessary conditions are complied with. Tissues should be handled rapidly to prevent autoxidation, and a control section is essential because, as has already been remarked, a positive result upon fresh tissue untreated with mercuric chloride might be due to any or all of several radicles and the means at our disposal do not allow of a discrimination between them.

This raises an interesting point. Unless the substrate can be shown to be lipid, there is no justification for considering the reaction to be due to plasmalogen and plasmal. A case in point is the medulla of the adrenal. The extremely powerful lipid-colorant Sudan black shows no lipid whatever in this region. Yet it colours, after Verne's technique, as strongly as the innermost region of the zona reticularis and the outermost of the cortex. Prolonged exposure of sections does result in the cortex becoming much more positive, but the medulla becomes more pale. Obviously, this is not a case of the Feulgen-Verne reaction of unsaturated lipoids; if it were, the medulla also would become more positive. But equally it cannot be said to be a true plasmal reaction because the medulla is negative with the technique given above. As Verne remarks (1929*b*), phenylhydrazine abolishes the reaction in both cortex and medulla. But phenylhydrazine is a strong base, and soaking tissues in strong bases reduces considerably their power of being stained by basic dyes. If this were the mechanism, then the nature of the colouring matter is in doubt, because it has the appearance of a product of Schiff's reagent, but, as we have seen, such products do not appear to act appreciably as dyes. Verne claims that it is due to aldehydic substances which diffuse from the cortex into the medulla. The complete proof of this would require that a definite reaction could be produced on extracts of the medulla made with lipid-solvents, and that this could be shown to be the same as that found in the reticularis. A mere suppression of the reaction by treatment with alcohol is not sufficient proof. Alcohol has many effects on tissues besides extracting some of the lipoids. There is, however, another possible explanation. Basic precipitates of mercuric chloride solutions will turn deep violet in the presence of Schiff's reagent. Now the colouring of adrenal sections fixed in mercuric chloride is brightest around the free edge of the cortex and the edges of the large fenestrae caused by vigorous fixation in the medulla. It is at least

possible that this distribution is due to unequal conditions of fixation. One may decide that these explanations are unlikely, and it is more reasonable to attribute the reaction to plasmal; but there is no histochemical proof. But where lipoids can be demonstrated in the tissue, and the reaction ceases when they are removed, and mercuric chloride exerts a profound and rapid effect, it is likely that plasmalogen and plasmal are responsible.

The techniques available for demonstrating plasmal histochemically are not completely satisfactory, the demands of the cytologist and chemist being at variance. The reaction can easily be studied macroscopically on small pieces of fresh organs dropped into the reagents. Short fixation is necessary for good results. As mercuric chloride is a very powerful precipitant, the best procedure is probably to fix very small pieces for a short time in a formaldehyde fixative, wash carefully, and carry out the technique on frozen sections in the way usually recommended. But pieces fixed and stained simultaneously according to the procedure given above should also be examined. The reaction in them is far more intense, and far more tissue elements are coloured. This is almost certainly the reason why the Feulgen school, working on fresh sections, found the plasmal reaction so widespread, while Verne, using fixed material, found it far less so. As liver appears to give only the feeblest reaction even when fresh, a piece of it can be carried through with the other tissues under investigation, to act as a general control on the method. It is not denied that a true plasmal reaction can be obtained on pieces fixed for several hours, but it is greatly reduced and may easily be confused with the beginnings of the Feulgen-Verne reaction.

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SUMMARY

1. Two different reactions have been confused under the name 'plasmal'. One, the true plasmal reaction, is due, as its discoverer Feulgen showed, to the liberation of higher aliphatic aldehydes from acetalphosphatides, the reaction taking place very rapidly under the influence of mercuric chloride, the products being coloured deeply with Schiff's reagent. The other reaction is due to the oxidation, by atmospheric oxygen, of double bonds in unsaturated fatty acid radicles. It is affected only slightly, if at all, by mercuric chloride, and increases with exposure of sections or pieces of tissue to atmospheric oxygen.

2. There is no completely satisfactory method for the histochemical demonstration of plasmal. Sections of fresh tissues are not usually suitable for cytological work, and fixation causes a reduction in intensity of the

reaction. Also, the permissible fixatives are not all of the highest quality. Short fixation in a formaldehyde fixative (e.g. formal-calcium) followed by careful washing is probably the best, but the results should be compared with those obtained by the direct reaction of small pieces of fresh tissue. A suitable technique is described.

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The Cell-Theory: a Restatement, History, and Critique

PART II

BY

JOHN R. BAKER

(From the Department of Zoology and Comparative Anatomy, Oxford)

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PROPOSITION II

Cells have certain definable characters. These characters show that cells (a) are all of essentially the same nature and (b) are units of structure.

THE essence of this proposition can most easily be grasped by considering what would be left of the cell-theory if it were omitted. We should then be in the same position as was Leeuwenhoek (1674), who, having found that a number of tissues consisted of ‘globules’, was not surprised to find the same structure in milk. This second proposition is concerned with the reasons for supposing that certain objects, called cells, are all to be regarded as strictly comparable with one another and not comparable with globules such as those of milk.

Very gradually, over a period of centuries, it came to be recognized that there is a fundamental living substance, the protoplasm; that this protoplasm commonly occurs in small masses, each provided with a nucleus; and that each of these masses is to some extent separated from its neighbours by a cell-membrane having special characters. Proposition II covers these discoveries and is also concerned with the reasons for supposing that cells are unitary components of organisms and that one cell corresponds with one other cell and not with several. The present paper deals with the discovery of protoplasm and the nucleus. The discussion of Proposition II will be continued in Part III of this series of papers.

The Discovery of Protoplasm

One of the most fundamental facts about cells is that they contain protoplasm as their characteristic constituent, and that, with some partial exceptions that are mentioned on p. 98, this substance never occurs except in

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cells or in objects formed by the transformation of cells. While allowing that the word protoplasm has no absolutely precise meaning, we must acknowledge that there are many substances of which it can be stated with certainty that they are not protoplasm, and that such substances occur commonly between cells, but never constitute cells; while the substance that exists in cells (and in transformed cells) and is called protoplasm has so many positive characters that it is impossible to suppose that we are lumping together under a single name utterly distinct mixtures of organic compounds. This is not the place to give a list of the physical and chemical properties of the substance; we are concerned here only to trace the history of the idea that the cells of plants and animals have a substance called protoplasm as their characteristic component.

The earliest observations and experiments on this substance were not made in connexion with cells. Trembley (1744) made a careful study of the protoplasm of *Hydra* without ever understanding the cellular nature of these animals. He was investigating the microscopical 'grains' (apparently the carotene-granules and nematocysts) that he had discovered. He noticed (p. 56) that when he had teased up a fragment of the body in a drop of water, some of the 'grains' remained bound together by 'une matière glaireuse' (literally, a substance resembling white-of-egg). Trembley stretched a fragment of the body between the points of a quill pen and saw the glairy substance spin out between them (p. 57). He was able to isolate this substance almost completely from the granules. He attributed the cohesion of the granules to the glairy substance. He was also able to stretch a tentacle and to obtain a microscopical view of the part of it lying between two 'grains' (nematocysts): this part consisted wholly of the glairy substance (pp. 63-4). He notes its *transparency* and *tenacity*, the latter being shown by the resistance of the tentacle against breaking when pulled. He attributes to it also the polyp's powers of contraction and expansion.

Duhamel du Monceau (1758, p. 26), in his study of the cellular tissue of plants, mentions the 'substance vésiculaire, ou cellulaire' that fills, as he says, the meshes of the net (i.e. the spaces enclosed by the cell-walls). He remarks that it contracts on desiccation and that it is sometimes coloured.

The discovery of cyclosis by Corti (1774, pp. 127-200) was to play an important part some three-quarters of a century later in leading microscopists to the opinion that the living substance of plants and animals is essentially similar (see p. 95). At the time, the circulation of protoplasm was only an isolated curiosity. Corti uses the name 'Cara' for the various species of freshwater plants on which his observations were made; these included *Chara* and perhaps also *Nitella*. He uses the name *Cara translucens minor, flexilis* for the species in which he first saw the circulation of granules in the long internodal cells.

Treviranus (1811, pp. 78-95) first saw cyclosis in 1803. His observations on this subject were made on *Hydrodictyon utriculatum* and *Nitella flexilis* (which he calls *Chara*), among other freshwater plants. It is clear that he

had no knowledge of Corti's discovery. He would appear to have confused cyclosis with the movement of reproductive cells set free by algae, as observed by other authors.

Treviranus calls the protoplasm of freshwater algae 'Gallert oder organische Materie'; he notices the granules in it and mentions that he has seen them also in the cells of the cellular tissue of plants. Indeed, he found each cell of this cellular tissue 'ganz ähnlich' to a segment (Glieder) of a conferva (1811, p. 78).

Brisseau-Mirbel (1815, p. 196) uses Grew's word 'cambium' more or less as we might say 'the protoplasm of meristematic tissues'. He describes it as a colourless mucilage that appears wherever new developments are going to occur. He did not understand that it was partitioned into cells, but considered that though a fluid, it contained the 'linéaments' of new structure. In his text-book of histology, Heusinger (1822, p. 41) uses the expression 'Bildungsgewebe (tela formativa)' roughly in the sense of what we should call protoplasm; but his style is reminiscent of Oken's *Naturphilosophie* and he does not give much precise information.

Dujardin (1835) was led to the study of protoplasm by his doubts as to the correctness of Ehrenberg's opinion that the food-vacuoles of ciliates are stomachs joined by an intestine. He was unable to see any tube joining one vacuole to another and his attention was thus directed to the intervening substance. He says that he would perhaps have abandoned these studies, if he had not solved the problem by the discovery of the properties of 'Sarcode'. 'I propose to give this name', he says (p. 367), 'to what other observers have called a living jelly—this glutinous, transparent substance, insoluble in water, contracting into globular masses, attaching itself to dissecting-needles and allowing itself to be drawn out like mucus; lastly, occurring in all the lower animals interposed between the other elements of structure.' It is remarkable that Dujardin at once seized upon most of the important physical characters of the substance he had just named. Indeed, one could hardly improve upon his description in a short statement, except by providing the numerical data that are available to-day. He found (pp. 367-8) that sarcode decomposes gradually in water; unlike albumen, it does not dissolve, but leaves a feeble, irregularly-granular residue. Potash hastens the decomposition; nitric acid and alcohol coagulate the substance and make it white. It spontaneously produces vacuoles within itself. It refracts light much less than oil does.

Dujardin studied sarcode not only in ciliates, but also in *Fasciola* and *Taenia*, in *Nais*, earthworms and other annelids, and in young larvae of insects. He seems generally to have used exudations from rents in tissues for his metazoan material.

Dujardin did not relate his sarcode to cellular structure. Various microscopists, however, began to make short remarks about the substance lying between the nucleus and the boundary of the cell. Valentin (1836), who studied it in nerve-cells, called it the 'Parenchym'. He said that it was 'for the most part a grey-reddish finely granular substance', though transparent

and clear as water in fishes (p. 138). He mentions the 'small, dispersed, separate, round particles' in the cytoplasm of various nerve-cells, and figures them (see especially his Figs. 45 and 49 of Tab. VII). These were almost certainly the vacuoles or spheroids that constitute the basis of the so-called Golgi apparatus, and he should presumably be regarded as the discoverer of this cytoplasmic element.

Schleiden (1838, pp. 143-5) seems to have used the word 'Schleim' in more or less the sense of plant protoplasm; but his attention was so much fixed upon the nucleus and cell-wall, and his ideas on the origin of cells so mistaken, that it is impossible to be sure. Certainly he did not believe his 'Schleim' to be an essential part of the cell, except in so far as the nucleus might be formed of it. He says that it occurs in irregular, granular forms without internal structure, and is stained brownish-yellow or brown by tincture of iodine. He seems to have thought that what we should call the cytoplasm of young cells was a watery fluid *containing* granules of Schleim.

Meyen (1839), like Dujardin, was led to the study of protoplasm by investigating the food-vacuoles of ciliates. Like Dujardin, he denied Ehrenberg's opinion that these animals have stomachs joined by an intestine: there are simply watery vacuoles (Höhlen) in a gelatinous substance. 'The true infusoria', he wrote, 'are bladder-like animals, the cavity of which is filled with a slimy, somewhat gelatinous substance' (p. 75). He mentions (p. 79) that similar vacuoles occur in the 'Schleim' of the cells of plants, particularly in the aquatic filamentous forms, but he is so much interested in the vacuoles that he omits to institute a comparison between the gelatinous substance of infusoria and the Schleim of plants.

Schwann (1839*a*) added little to knowledge of the living substance. He mentions (p. 12) that the cells of the notochord of the frog-larva contain a colourless, homogeneous, transparent substance, which, he says, does not become cloudy at the temperature of boiling water; and he describes the contents of ganglion-cells (p. 182) as being a finely granular, yellowish substance. He gives some account (p. 45) of the 'strukturlose Substanz' of organisms; but this was the supposed Cytoblastem or substance in which cells originate, not the substance of cells themselves. Schwann states specifically (p. 209) that the substance that comes to surround the nucleus in the developing cell is different from the Cytoblastem. He says little about its characters, however, beyond mentioning that it is sometimes homogeneous and sometimes granular.

The first attempt to generalize about the properties of the living substance of plant and animal cells was made by Purkinje on 16 January 1839 at a meeting of the Silesian Society for National Culture. A report on his address was published the following year (Purkinje, 1840*a*). The intrinsic value of his remarks, and the fact that he used the word 'Protoplasma' for the first time in its scientific sense, make it necessary to reproduce a considerable part of what he said. The word Protoplasma had long been used in religious writings in the sense of the 'first created thing'; but it is a surprising—indeed

an astonishing—fact that in introducing the term into science, with a very particular and important meaning, he gives no indication that it was not already in current use in this field. He reserves the word 'Zellen' for cells that have distinct cell-walls, using 'Kügelchen' and 'Körnchen' for those that have not. He uses the word 'Cambium' in the same sense as did Brisseau-Mirbel. He wrote as follows:

'In plant-cells the fluid and solid elements have separated completely in space, the former as the inner, enclosed part, the latter as that which encloses it. In the animal development-centre, on the contrary, both are still present in mutual permeation. The correspondence is most clearly marked in the very earliest stages of development—in the plant in the cambium (in the wider sense), in the animal in the Protoplasma of the embryo. The elementary particles are then jelly-like spheres or granules, which present an intermediate condition between fluidity and solidity. With the advance of development the animal and plant structures now diverge from one another; for the former either tarries longer in the embryonic condition or remains stationary in it throughout life, while in the latter on the contrary the hardening process and the separation of the solid and the fluid progress more rapidly, and come to light first in cell-formation and then in the formation of vessels.'

It will be noticed that although he applies the word Protoplasma only to the substance of the embryonic cells of animals, yet he clearly realizes the correspondence of this substance with that of the adult cells of animals and of the meristematic cells of plants. In the case of the adult plant cell, he regards what we call simply the protoplasm as constituting the fluid part of his Protoplasma, the solid part having separated out as the cell-wall. In another paper, published in the same year, Purkinje (1840b) follows up these ideas by claiming that there should be a 'Körnchentheorie' as opposed to the cell-theory of Schwann, since plants and animals originate from simpler elementary granules, which in plants become changed into cells, while in animals they either remain as they were or change into various forms of fibres. He does not use the word Protoplasma in this paper, but the idea of a substance common to plant and animal cells is implicit in what he writes.

Jones (1841) denied, like Dujardin, that the 'internal sacculi' of ciliates are connected by an intestine (pp. 56–8). He states that the lowest animals consist of a 'gelatinous parenchyma' (p. 6). He speaks of a 'semifluid albuminous matter' loosely connecting the green granules of *Hydra* (p. 21).

Kützing (1841) helped to direct attention towards the protoplasmic part of plant cells, but unfortunately used a confusing terminology. He claimed that each cell of a conferva consists of three elementary parts: the outer 'Gelinzelle', the 'Amylidzelle', and the 'Gonidien'. The first, from his description, was clearly the cell-wall. The second was what von Mohl was later to call the Primordialschlauch, that is the layer of protoplasm lining the cell-wall on the inside. He describes the Amylidzelle as being coloured

brown by iodine; weak acids, alcohol, and drying cause sudden contraction, which cannot be reversed by soaking in water. Kützing made the mistake of supposing that caustic potash converts this layer into starch. The third elementary part was the granular material enclosed by the Amylidzelle (starch-grains, &c.).

A considerable advance was made by Nägeli (1844), who found (pp. 90-1) that there is a slightly granular, colourless 'Schleimschicht' under the whole of the inner surface of the cell-wall of the fully formed cells of green algae and of some fungi. The chloroplasts and starch-grains are attached to it. The whole of the rest of the cells is filled with a water-clear fluid. Nägeli understood that his Schleimschicht corresponded to the Amylidzelle of Kützing, but he objected to the latter's name, firstly because it is not a Zelle (in the sense of 'box'), and secondly on chemical grounds (p. 96). The Schleimschicht, he found, consists of granular slime, which earlier filled the whole cavity of the cell and now lies just within the cell-wall. Its outer surface is smooth, but towards the interior of the cell it forms rather irregular projections. The name 'cell', he insists, is not suitable for such a structure. The Schleimschicht is coagulated by alcohol, weak acids, and water; these are the properties of nitrogenous plant-slime. It is coloured brown by iodine, and it is not changed into starch by potash, as Kützing had said.

These researches of Nägeli to a large extent forestalled the more famous work of von Mohl, who gave the name of Primordialschlauch, or utriculus primordialis, to the protoplasmic layer that lines the inside of the cell-wall of plants (1844, col. 275). This primordial utricle clearly corresponds to the Amylidzelle of Kützing and the Schleimschicht of Nägeli. Von Mohl's term conveys clearly his realization that the cell-wall is not the primary or fundamental part of the cell. He mentions (col. 276) that when a nucleus is present, it lies in the primordial utricle, generally attached to its inner wall; when the nucleus is centrally situated, it is connected to the primordial utricle by slimy threads. The cell-wall stains blue with iodine, while the primordial utricle stains yellowish-brown.

Two years later von Mohl (1846) reintroduced the word 'Protoplasma'. He was quite obviously unaware that Purkinje had already used the word in the same sense. The importance of von Mohl's remarks on this subject justifies rather a long extract. He remarks (col. 73) that if we study a young plant cell, we never find that it contains a watery cell-sap: a viscous, colourless mass, containing fine granules, is dispersed through the cell and is aggregated especially in the vicinity of the nucleus. He thought that this substance was present before the nucleus appeared. 'As has already been remarked,' he writes (col. 75), 'wherever cells are going to be formed this viscous fluid precedes the first solid structures that indicate the future cells. We must further suppose that the development of structure in this substance is the process that initiates the formation of the new cell. For these reasons there may well be justification if, for the designation of this substance, I propose in the word *Protoplasma* a name based on its physiological function.'

(In this translation I have used the expression 'development of structure' to convey the meaning of von Mohl's word 'Organisation'.)

In a footnote von Mohl mentions that Schleiden used the word 'Schleim' in the same sense. Von Mohl objected to this word because it was already used on the one hand loosely for any substance whatever that is of a slimy consistency, and on the other hand in a restricted sense as a synonym for mucus.

He describes (col. 76) how in young cells the nucleus always lies at the centre, surrounded by protoplasm. He proceeds (cols. 77-8) to an account of the origin of the cell-sap. 'Irregularly distributed spaces form in the protoplasm, which fill themselves with watery sap. . . . The older the cell becomes, the more these spaces filled with watery sap increase in size in comparison with the mass of the protoplasm. As a consequence the spaces that have been described flow together into one another.'

It will be allowed that von Mohl had now arrived at a remarkably exact idea of the general plan of a plant cell.

The next necessary step was the discovery that protoplasm is the fundamental constituent of the cells of animals as well as of plants. It might be thought that since the word had first been applied to animal tissues, this step would have been an easy one; but Purkinje's ideas had not received the recognition that was their due, nor had his word 'Protoplasma' been accepted by students of animal cells. The ground gained by Purkinje required to be recaptured.

To Ecker (1848) is due the recovery of the idea that there might be a fundamental substance common to animals of all grades of structure. His object was to discover what there was in lower animals corresponding to the contractile substance of higher animals. He felt that Dujardin's work on sarcode had been disregarded by most histologists. There had been a mistaken tendency to look for parts corresponding to those of the higher animals in the bodies of the lower. 'The body of the Infusoria . . .', he writes (p. 221), 'consists throughout of a simple, homogeneous, half-fluid, jelly-like substance, in which neither cells nor fibres are perceptible—a substance that is sensitive and contractile and in which the essential properties of the animal body are thus not yet confined to particular tissues.'

Ecker concentrated a good deal of his attention on *Hydra*, in which animal he failed to notice the muscular bases of the epithelial cells. He gave the name 'ungeformte contractile Substanz' to the sarcode of Infusoria and the living material of *Hydra*. He found that both were albuminous, soft, either wholly homogeneous and transparent or finely granular; both contained bladder-like spaces or vacuoles; both were in the highest degree elastic and contractile; both insoluble in water, though altered by it; both soluble in potassium hydroxide but hardened and contracted (so he said) by potassium carbonate (pp. 237-8). He claimed to have traced the development of the true striped muscle of the *Chironomus* larva from a completely homogeneous, fibreless, contractile substance.

Ecker's work was important chiefly for its influence on Cohn (1850), who listed the properties of the contractile substance of animals as described by Dujardin and Ecker and then went on to show that this substance was the same as the protoplasm of plant cells. His words (pp. 663-4) are of the utmost importance for the history of the discovery of protoplasm, and must be quoted in full: '*But all these properties are possessed also by protoplasm, that substance of the plant cell which must be regarded as the chief site of almost all vital activity, but especially of all manifestations of movement inside the cell. Not only does the optical, chemical and physical behaviour of this substance correspond with that of sarcode or the contractile substance (which I had the opportunity to study in the Infusoria, Hydra, and Naids)—in particular, both substances are very rich in nitrogen, are browned by iodine and contracted by stronger reagents—but also the capacity to form vacuoles is inherent in plant protoplasm at all times. . . .*

'Hence it follows with all the certainty that can generally be attached to an empirical inference in this province, that the protoplasm of the botanists and the contractile substance and sarcode of the zoologists, if not identical, must then indeed be in a high degree similar formations.

'Accordingly, from the foregoing point of view, the difference between animals and plants must be put in this way, that in the latter the contractile substance, the primordial utricle, is enclosed within a rigid cellulose membrane, which allows it only an internal mobility, normally expressing itself in the phenomena of circulation and rotation—while in the former this is not so.'

Despite this last paragraph, Cohn did not regard the cell-wall as a fundamental part of the plant cell, for he wrote (pp. 655-6): '*In general I comprehend under the expression "primordial cell" that form of the primordial utricle which assumes the aspect of a cell and appears either altogether devoid of a rigid cell-membrane, or independent and isolated from one.'*

It would be difficult to exaggerate the importance of the contribution to our knowledge of protoplasm made by Cohn in the passage just quoted. The contributions made subsequently by Unger, Schultze, Haeckel, and their contemporaries, were amplifications of ideas first formulated by Cohn.

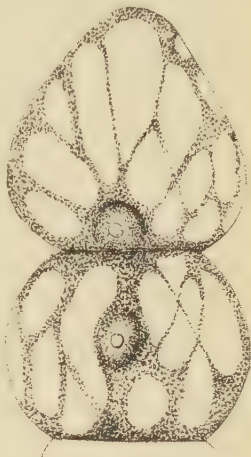
Von Mohl now devoted a book (1851) to the characteristic features of the anatomy and physiology of the plant cell. He remarks (pp. 42, 44) that the protoplasm constitutes a relatively small part of the fully developed plant cell, owing to the large size of the spaces occupied by the cell-sap, which does not mix with the protoplasm. It is difficult to be certain of the exact meaning attached by von Mohl to his word 'Primordialschlauch'. Did he mean the whole of the protoplasmic layer that lies below the wall of the plant cell, externally to the vacuole? or did he mean only the external membrane of this protoplasmic layer? There are remarks on pp. 41-4 which suggest that he was referring to the cell-membrane in the modern sense; but other passages in his writings do not confirm this view, and he does not figure the cell-membrane separately from the protoplasm. He does, however, give a remarkably good figure of typical plant cells, reproduced here as Text-fig. 1.

Remak (1852, p. 53) now adopted von Mohl's botanical word 'Protoplasma' in referring to the substance of the egg-cell and embryonic cells of animals.

The course of progress was now briefly interrupted by an extraordinary episode. T. H. Huxley (1853) made an attempt to discredit not only the view that protoplasm is the fundamental living substance, but also the cell-theory as a whole. He recognized two constituents of tissues: the endoplast (which we should call the protoplasm) and the periplast (intercellular material). His object was to show that life depends primarily upon the intercellular material. 'So far from being the centre of activity of the vital actions', he writes (p. 306), 'it [the endoplast] would appear much rather to be the less important histological element. The periplast, on the other hand, which has hitherto passed under the names of cell-wall, contents, and intercellular substance, is the subject of all the most important metamorphic processes, whether morphological or chemical, in the animal and in the plant.' The endoplast, he says (p. 312), 'has no influence nor importance in histological metamorphosis.' 'We have tried to show', he says (p. 314), 'that they [the cells] are not instruments but indications—that they are no more the producers of the vital phenomena, than the shells scattered in orderly lines along the sea-beach are the instruments by which the gravitative force of the moon acts upon the ocean. Like these, the cells mark only where the vital tides have been, and how they have acted.'

In an important paper Unger (1855) brought strong support to the views that had been formulated five years before by Cohn. After considering the properties of plant protoplasm, and especially its movements, he concludes (p. 282): 'So all this suggests that protoplasm must be regarded not as a fluid, but as a half-fluid contractile substance, which is above all comparable to the sarcode of animals, if indeed it does not coincide in identity with the latter.' Schultze (1858) next described the movement of granules in marine diatoms and compared it with that seen in *Noctiluca* and in the pseudopodia of *Gromia*, Foraminifera, and Radiolaria. In his oft-quoted paper of 1861, which will be considered further in Part III of this series of papers, he mentions that Remak's adoption of von Mohl's word 'Protoplasma' for the substance of animal cells has not been generally copied, and says that he himself will use it henceforth. His example was probably influential.

Schultze's account of the movement of granules in protoplasm was attacked by Reichert (1862*a* and *b*), who claimed that the appearance was illusory. Schultze (1863) had little difficulty in showing that Reichert was mistaken. He proved that the granules of *Gromia* and other rhizopods



TEXT-FIG. I. Von Mohl's figure of typical plant cells. The figure represents part of a hair (probably a staminal hair) of *Tradescantia Selowii*. (Von Mohl, 1851, Tab. I, Fig. 7.)

are real and that they display characteristic movements during life. He also studied the hairs on the stamens of *Tradescantia* and the parts of other plants in which cyclosis is observed. He found a remarkable agreement. 'The movements in the protoplasm of plant cells', he writes (p. 65), 'resemble those in the pseudopodia of the Polythalamia [Foraminifera] so closely, that when the arrangement of the protoplasm is of the kind that occurs, for example, in the cells of the staminal hairs of *Tradescantia*, no difference between the two kinds of movement is to be discovered.' Schultze also showed that chemical and physical influences had similar effects on plant and animal protoplasm.

The pseudopodia of Foraminifera and Radiolaria lent themselves particularly well to studies of protoplasm. Haeckel (1862) made a careful investigation of its properties as revealed in the latter group. He used his powerful influence in support of Cohn and his successors. He wrote (Häckel, 1868, p. 108): 'The *protoplasm* or *sarcode theory*—the doctrine that the albuminous contents of animal and plant cells (or more correctly, their "cell-substance") and the freely motile sarcode of the rhizopods, myxomycetes, protoplasts, etc., are identical and that in both cases this albuminous substance is the originally active substrate of all the phenomena of life—may well be characterized as one of the greatest and most influential achievements of the newer biology.' After paying tribute to the work of Cohn, Unger, and Schultze, he continues (p. 109): 'I have myself striven for a number of years to support and extend this doctrine by numerous observations.'

Meanwhile a strange figure had entered the field. Beale was independent to the point of perversity; he insisted on using a private terminology of his own; and his writings were marred by their polemical character. Had he understood better how to integrate his own discoveries with those of others, he would have made greater contributions to research on protoplasm. Beale first made his ideas public in a series of lectures to the Royal College of Physicians in 1861 (Beale, n.d.). He distinguished between *germinal* and *formed* matter. The former, to which he ascribed the power of infinite growth, evidently corresponds to protoplasm, while the latter is the inter-cellular material. He regarded an affinity for carmine as a particularly striking character of the germinal matter. Beale's chief interest was in the synthetic function of the germinal matter, and his important contributions to this subject will be discussed under the heading of Proposition IV. The nucleus was for him the quiescent part of the germinal matter. Eventually he accepted the word protoplasm and wrote a book with that name (Beale, 1892); but, wayward to the end, he remarks in it that 'Nowhere in the world is the essential living element a "cell"' (p. 203).

Brücke (1862) brought a new insight into protoplasmic studies. He insisted (p. 386) that protoplasm has 'Organisation'. He denied (pp. 401-2) that it is either solid or fluid. He objected to its being called a slimy or jelly-like substance, for he thought that this was like the description of a medusa as a gelatinous mass by someone who was ignorant of its organization. The

cell-contents must have a complex structure in order to be able to perform the vital activities. His argument was largely deductive and seems to have had little influence on his contemporaries; but it foreshadowed the outlook of a later generation.

Although the Foraminifera and Radiolaria were well adapted for research on the living substance, yet the wholly protoplasmic nature of the Mycetozoa, combined with their fairly large size and ready availability in inland laboratories, made them of predominant importance. In the face of organisms which, in their active, plasmodial phase, contain no 'periplast' whatever, it was impossible any longer to maintain such views as had been put forward by Huxley in 1853. It was this that gave special importance to de Bary's careful study of the group. His description of the protoplasm of Mycetozoa (1864, p. 41) deserves quotation. 'The ground-substance always presents itself as a colourless, translucent, homogeneous mass, exactly similar to the homogeneous contractile substance that is known in the body of amoebae, rhizopods, etc., and is designated as *sarcode*, *unformed contractile substance*, and latterly, like the component of plant-cells which is in many respects analogous, as *protoplasm*. . . . As for its chemical character, rose-red colouring with sugar and sulphuric acid and with Millon's reagent, together with yellow colouring by iodine, indicate a rich content of albuminous substances. Alcohol and nitric acid cause coagulation; in acetic acid the substance becomes thin (blass) and transparent; in potassium hydroxide solution, even when dilute, it dissolves; the same occurs in potassium carbonate solution, though often after the first action of this reagent has produced a contraction.' It will be agreed that this is a remarkably exact short description of protoplasm.

Kühne (1864) brought strong evidence from many sources of the close similarity of plant and animal protoplasm. He studied the living substance in *Amoeba*, *Actinophrys*, *Didymium* (a mycetozoan), in the cells of the connective tissue and cornea of the frog, and in those of the staminal hairs of *Tradescantia*. He observed protoplasmic movements and noted the effects of reagents, of temperature changes, and of the passage of an electric current. He obtained protoplasm from staminal hairs (pp. 100-1) and was so struck by its resemblance to that of *Amoeba* that he called particles of it 'vegetabilische Amöben'.

Huxley's rhetoric was now to be used once more on the subject of protoplasm. Without giving any indication that he had reversed his opinions or had made any observations or experiments that could cause him to do so, he plunged into powerful support of the protoplasm theory. The occasion was a Sunday evening address given in the Hopetoun Rooms, Edinburgh. According to the careful report given in the *Scotsman* (Huxley, 1868, p. 7), he described protoplasm as 'the bases [*sic*] of physical life'; the expression 'the physical basis of life' first appeared in print as the title of his article in the *Fortnightly Review* (Huxley, 1869), which followed closely the Edinburgh address. 'Beast and fowl,' he wrote (pp. 134-5), 'reptile and fish, mollusk,

worm and polype, are all composed of structural units of the same character, namely, masses of protoplasm with a nucleus. . . . What has been said of the animal world is no less true of plants. . . . Protoplasm, single or nucleated, is the formal basis of all life.' The discovery had been made by others: Huxley's contribution was first opposition and then a phrase.

It is to be noticed that the essential similarity between the living matter of plants and animals was discovered by examination of the ground cytoplasm before the existence of mitochondria in both was recognized.

A relatively small point remains. Russow (1884, pp. 578-9) discovered that in the medullary rays of certain plants there exists intercellular material that colours like protoplasm with iodine and dyes. In *Acer* he found thin threads connecting this intercellular with cellular protoplasm. In the same year Fromman (1884) claimed that protoplasm exists in the intercellular spaces of the hypocotyl of *Ricinus*. Like Russow, he said that it reacted to iodine and dyes in the same way as cellular protoplasm. He stated that it often contains single starch grains and small chloroplasts. Intercellular material had already been studied in the cotyledons of the pea by Tangl (1879), who regarded it, however, as secreted matter. The corresponding intercellular substance in the cotyledons of *Lupinus* was seen and figured by Michniewicz (1903), who later saw bridges connecting it with cellular protoplasm (1904). The intercellular material in the cotyledons of *Lupinus* was studied in considerable detail by Kny (1904a). He found that reactions for proteins were positive. He concluded from the bleaching of methylene blue solutions and the blue coloration with guaiacol and hydrogen peroxide that the intercellular material respire. Indeed, he found that it showed the same reactions as cytoplasm in all respects, except that studies with proteolytic enzymes suggested that it contained more protein. His general conclusion was that the substance was in fact intercellular protoplasm. In a second paper (Kny, 1904b) he showed that it is connected with the cells of the cotyledons by narrow bridges. Like Russow, he found that intercellular protoplasm may contain small starch grains.

This subject would be of considerable importance from the point of view of the cell-theory if it could be shown that intercellular protoplasm ever exists in the absence of any connexion with nucleated cells; but this would not appear to be so. The tissues of animals do not provide any close counterpart to the intercellular protoplasm that occurs occasionally in plants.

The Discovery of the Nucleus

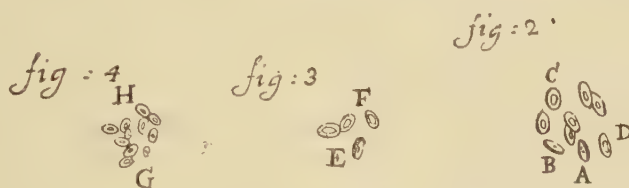
Some of the older botanical writers (e.g. Balfour, 1854) call the nucellus of the ovule the nucleus. This may give rise to misunderstanding. It was stated by Meyen (1839, p. 250), for instance, that both Grew and Malpighi saw the 'Kern' of the 'Eychen' (ovule) of plants. On the same page he uses the word 'Nucleus' as equivalent to 'Kern'. Reference to the relevant parts of Grew's and Malpighi's works shows that there is no question of the

object named being a nucleus in the modern sense (see Grew, 1682, p. 210 and Tab. 82; Malpighi, 1687, p. 71, and Fig. 233 on Tab. xxxvii).

Nuclei were in fact first seen by Leeuwenhoek, whose description of them is contained in a letter sent to the Royal Society in 1700 (see Leeuwenhoek, 1702, p. 556; Leeuwenhoek, 1719, p. 219). The discovery was made in the red blood corpuscles of the salmon. His description of the figure made by his draughtsman (see Text-fig. 2) is as follows:

'Fig. 2 ABCD represents the oval particles of the Blood of a Salmon that weighed above thirty pound.

'AB represents the particles that appeared flat and broad, but did not face the eye directly.



TEXT-FIG. 2. The earliest figure of the nucleus. Red blood corpuscles of the salmon and 'Butt', as represented by Leeuwenhoek's draughtsman. The original numeration of the figures has been retained so that it may correspond with Leeuwenhoek's description as reproduced in the text. (Leeuwenhoek, 1702, Plate opposite p. 220.)

'Those about c came straight upon the eye, and for the most part had a little clear sort of a light in the middle, larger in some than in others, which the Engraver has done his utmost to imitate.'

There can be no doubt that the 'little clear sort of a light' (*lumen* in the Latin version (Leeuwenhoek, 1719)) was the nucleus. Leeuwenhoek also saw nuclei in the blood of a small fish which he calls 'Butt' (1702) or 'Botje'. 'Butt' was a general term in English for flat-fish; in modern Dutch 'bot' is the flounder. Figs. 3 and 4 in Leeuwenhoek's plate (reproduced here as Text-fig. 2) represent the red blood corpuscles of this fish. He refers to the nuclei as 'little shining spots' (1702, p. 557).

Nuclei were seen by some of the early students of Protozoa. Writing to Réaumur in 1744 Trembley gave some sketches of *Stentor* that show the moniliform macronucleus clearly (see Trembley, M., 1943, p. 207). The same observation was made on *Stentor* by Müller (1786, Tab. xxxvi, 8), who calls the macronucleus 'Series punctorum pellucidorum' (p. 262). Müller appears also to have seen the macronucleus in a species of *Colpoda* and a few other ciliates, but there is no indication that he recognized its homology in the different forms. Much later Ehrenberg (1838) saw the macronuclei of many ciliates (*Amphileptus*, *Nassula*, *Chilodonella* ('*Chilodon*'), *Paramecium*, *Spirostomum*, *Stentor*; see his Plates xxiii, xxiv, xxxvi, xxxix), but did not understand their nature. It is convenient to mention Ehrenberg in connexion with this early stage of the history, because his work on the subject was not related to the nuclear research of his own period, for in

accordance with his opinion that the ciliates were 'vollkommene Organismen', he regarded their macronuclei as male reproductive glands (see, for example, pp. 262, 332, 352).

Hewson (1777) illustrated the nuclei in the red blood corpuscles of many vertebrates—birds, viper, slow-worm, frog, and fishes—and also saw them in the turtle. He made the understandable mistake of suggesting their presence in his figures of the red blood corpuscles of mammals. He was the first to see the nucleus in the blood corpuscle of an invertebrate. 'If one of the legs of a lobster be cut off,' he writes (p. 40), 'and a little of the blood be catched upon a flat glass, and instantly applied to the microscope, it is seen to contain flat vesicles, that are circular like those of the common fish, and have each of them a lesser particle in their centre, as those of other animals.'

Fontana (1781) would appear to have been the first to have seen nuclei in tissue-cells other than those of blood. He made a microscopical study of the slippery substance that coats the skin of eels and described the 'globules' or 'vesicules' contained in it, which were almost certainly epithelial cells derived from the epidermis. 'One saw', he says (p. 276), 'a little body internally, situated in different parts of each globule.' He means that there is not a characteristic position for the little body within the cell. His figures (e.g. Fig. 9 of Plate 1 in Vol. 2) strongly suggest that the 'petit corps' was the nucleus. Indeed, Fig. 10 shows what seems to be a nucleolus within the nucleus. Fontana says, 'The vesicle *a* in Fig. 10 represents one of the vesicles of Fig. 9, in which one observes an oviform body [the nucleus], having a spot (*tache*) in its interior.' This '*tache*' is probably the earliest illustration of a nucleolus. A life-like view was obtained of these cells because they lay in a medium of suitable osmotic pressure. The early observers were accustomed to tease up tissues in water, in preparation for microscopical examination; and it was only when nature provided a suitable medium and thus made the addition of water unnecessary that good views of tissue-cells were obtained.

When Purkinje discovered the germinal vesicle of eggs, he had no means of knowing that there was any correspondence with the 'lumen' seen in the blood corpuscles of fishes or with the 'petit corps' in the epidermal cells of eels. The discovery was not widely known until five years after it had been made. The Faculty of Medicine at Breslau had decided to congratulate Blumenbach in 1825 on the fifteenth anniversary of his taking his doctor's degree, and they wished to send him an original scientific paper to mark the occasion. Purkinje's offer to write a memoir for this purpose was accepted. It was made available to the world at large in 1830 (see Purkinje, 1830 and 1871). The germinal vesicle of the hen's egg was described in this memoir as follows (1830, p. 3):

'Thus the scar [germinal disk] of the ovarian egg contains a special part, peculiar to itself, a vesicle of the shape of a somewhat compressed sphere. This vesicle is limited by a very delicate membrane and filled with a special

fluid, perhaps connected with procreation (for which reason I might call it the germinal vesicle); it is sunk into a white breast-shaped projection composed of globules and perforated in the middle.' This remarkable passage contains the earliest mention of the objects later to be named the nuclear membrane and nuclear sap.

Coste (1833, col. 243) showed that the egg of the rabbit contains a vesicle corresponding to that discovered in the hen's egg by Purkinje. He later published a monograph (Coste, n.d.) giving figures of the nucleus of the rabbit's egg. In the description of his Fig. 2 he labels the nucleus 'vesicle analogous to that which Purkinje has demonstrated in birds'. Bernhardt (1834), who knew of Coste's work, found the nucleus, or 'vesicula prolifera' as he called it, in the egg of ruminants and of the rabbit, squirrel, bitch, cat, mole, and bat. He gives figures showing the nucleus in the eggs of several of these, and remarks that with certain precautions even a 'tiro' could see it. He says (p. 27) that it is round or nearly round or oval and has a sharp outline. The contents are fluid.

Meanwhile, nuclei had been discovered in plant cells. Bauer made drawings in 1802 which showed them in the cells of the loose tissue lining the canal of the stigma of the orchid, *Bletia Tankervilleae*. These drawings were unfortunately not published until much later (Bauer, 1830-8; see Tab. VI). Nuclei were probably seen from time to time in plant tissues without anyone guessing that they had any general significance. For instance, Meyen (1830, Plate III) shows what look like nuclei in the pith of the stem of *Ephedra*, though he himself regarded them as consisting of resin-like material. Brown, who knew of Bauer's drawing of *Bletia*, was the first to recognize that the nucleus is of more than sporadic occurrence, and it was he who coined the name by which this part of the cell has been known ever since. His words are as follows (Brown, 1833, pp. 710-11): 'In each cell of the epidermis of a great part of this family [Orchidaceae], especially of those with membranaceous leaves, a single circular areola, generally somewhat more opaque than the membrane of the cell, is observable. . . . This areola, or nucleus of the cell as perhaps it might be termed, is not confined to the epidermis, being also found . . . in many cases in the parenchyma or internal cells of the tissue. . . . I may here remark, that I am acquainted with one case of apparent exception to the nucleus being solitary in each utriculus or cell.' Brown also saw the nucleus in various cells of Liliaceae, Iridaceae, and Commelinaceae, and in a few cases also in the epidermis of dicotyledons.

The nucleolus, which had been recorded by no observer since Fontana, was now discovered by Wagner (1835) in the oocytes of various animals (*Ovis* (Fig. 2 on Tab. VIII), *Salmo*, *Phalangium*, *Anodonta*, *Unio*). He called it the Keimfleck or macula germinativa. The recognition of the nucleolus was important, because it helped in the identification of nuclei.

From 1836 onwards reports came repeatedly of the existence of nuclei in animal cells. Purkinje (1836) announced that the Körnchen (cells) covering the choroid plexus (apparently of man) are each provided with a small

'Körperchen'. Valentin (1836a, p. 97) introduced the word nucleus into the literature of animal cytology. Writing of the cells of the epithelium covering the vessels of the choroid plexus of the brain, he says: 'But each of them contains in the middle of its interior a dark, round kernel (Kern), a structure that reminds one of the nucleus that occurs in the plant kingdom in the cells of the epidermis, of the pistil and so forth.' Valentin classifies the epithelia, distinguishing those in which the cells are nucleated from those in which (as he supposes) they are not (p. 96). In a passage of quite extraordinary interest he makes a careful comparison between the egg and the nerve-cell. The latter he calls the formative sphere (Bildungskugel). 'But in what an astonishing way', he exclaims (pp. 196-7), 'does the basic idea of the form of the unfertilized egg correspond with the basic idea of the structure of the formative spheres!' He compares the membrane of these cells with the vitelline membrane, their 'Parenchym' (cytoplasm) with the early yolk, and their nucleus with the germinal vesicle; and he says that a 'Keimfleck' (nucleolus) occurs in both. In a second paper published in the same year (Valentin, 1836b), he again uses the word nucleus, stating (p. 143) that every cell without exception in the epithelium of the conjunctiva of man contains one. He mentions also that the nucleus itself here contains 'a perfectly spherical particle'.

The year 1837 saw the publication of a book of the first importance by Henle (1837), who now described nucleated cells in very diverse human tissues, including even the skin of the glans penis. He uses the word 'Cylindri' when referring to cells of columnar epithelium, but elsewhere uses 'Cellulae'. In describing his Fig. 4 he refers to the 'Cellulae nucleatae' of the human conjunctiva. He illustrates the nuclei of the epithelium of the trachea particularly clearly (Fig. 10). He mentions (p. 4) that the nucleus sometimes contains granules. It is not too much to say that this work, with that of Valentin (1836a), marks the beginning of an epoch in cytology, the epoch of the *nucleated cell*. Purkinje's name for the nucleus of the egg was, however, not readily relinquished. Siebold (1837) noticed the nuclei in the eggs and blastomeres of nematodes, but called them the 'Keimbläschen' and the 'Purkinjesche Bläschen' in the former case (p. 209) and 'hellen Flecke' in the latter (p. 212). He calls the nucleolus of the egg the 'Keimfleck'. These names, with their counterparts in other languages, persisted long afterwards, even when the homologies of the objects named were well understood.

The year 1838 brings us face to face with Schleiden and Schwann. To assess their significance in the advancement of cytology is a difficult task for the historian, and a task that has often been lightly undertaken. Too much credit has undoubtedly been given to them by some, and a reaction against exaggerated praise has produced a literature of rather superficial belittlement. It is necessary to realize in what field their chief contributions lay. They lay exactly here, in the part of this second proposition that is concerned with the nucleus. If there had been no Schleiden and no Schwann there would have been considerable delay in the general realization by

biologists that the possession of a single nucleus is a characteristic feature of most of the cells of animals and plants. Their work, taken together, provided most powerful evidence that there is a correspondence or homology ('*Uebereinstimmung*', Schwann called it) between the cells of the two kinds of organisms. Their ideas were far from being so original as has often been supposed; for Schleiden had his precursor in Brown, and Schwann in Purkinje, Valentin, and Henle. The three latter had made great advances in animal cytology during a period in which Brown's work on plant cells was scarcely being followed up, and Schleiden's contribution, therefore, represented a more sudden advance than Schwann's; and Schleiden was also ahead of Schwann and communicated his ideas to him in conversation. Indeed, one of Schleiden's most important functions was to act as a stimulus to Schwann: for one can scarcely read the writings of the two men without realizing that Schwann had the greater mind and made much the more massive contribution. These facts stand out even if we deliberately ignore the polemical character of much of Schleiden's writings. It must be allowed that Schleiden had too much influence on Schwann, for the latter took over, without sufficient investigation, his erroneous views as to the origin of cells. That, however, is beside the point for the present: we are here concerned with the great influence of these two men in getting the nucleated cell recognized as the fundamental building-stone of most organisms.

Schwann tells us (1839*a*, p. 8) that during the course of his work on the nerves of the tadpole of the frog he saw the cells and nuclei (Kerne) of the notochord. In his report on the subject (Schwann, 1837) he said nothing about the notochord; but the image of the notochordal cells remained in his mind. 'One day, when I was dining with Mr. Schleiden,' he tells us (Schwann, 1884, p. 25), 'this illustrious botanist pointed out to me the important role that the nucleus plays in the development of plant cells. I at once recalled having seen a similar organ in the cells of the notochord, and in the same instant I grasped the extreme importance that my discovery would have if I succeeded in showing that this nucleus plays the same role in the cells of the notochord as does the nucleus of plants in the development of plant cells.' The two scientists repaired at once to the anatomical institute in Berlin in which Schwann worked. Here they examined together the nuclei of the notochord, and Schleiden recognized the close resemblance to the nuclei of the cells of plants.

Neither had yet published. Schwann was the first to do so, but it will be convenient to begin with Schleiden's contribution, the '*Beiträge zur Phyto-genesis*' (1838). It is difficult to escape from a sense of disappointment on reading Schleiden's paper. There is nothing about a 'cell-theory' in it; it is solely concerned with plants; and it contains a great deal of error in connexion with the origin of cells, together with much that is of secondary interest. In one respect, however, it was of first-rate importance: the regular occurrence of nuclei in the young cells of phanerogams was here for the first time demonstrated. Schleiden thus focused attention on the nucleus as a

characteristic component of the cell. He was also the first to discover the nucleolus of plant cells, without realizing that it corresponded with the 'Keimfleck' already known in both germinal and somatic cells of animals. He calls it a small body (Körper, p. 141), but does not name it. He regards it as 'consistenter' than the rest of the nucleus. He shows it in several figures (see especially Fig. 25 on Tab. III). Beyond all this, Schleiden produced some ideas on cellular individuality, which will be considered under Proposition V.

Schwann's great contribution was his massive array of evidence that there is an 'Uebereinstimmung' between the cells of plants and animals. He himself concentrated upon the latter, relying on the researches of Schleiden for his knowledge of plant cells. We have already seen in the discussion of Proposition I that others had previously suggested such a correspondence between the plant and animal cell; but Schwann was struck with enormous force by the fact that each contains a corresponding object, the nucleus, itself containing a corresponding organelle, the nucleolus, which he called the 'Kernkörperchen'. It seems clear that he reached his conclusions from his own studies of animal cells and from discussion with Schleiden, before he knew of the discovery by Purkinje, Valentin, and Henle that the nucleated cell is a common constituent of animal tissues. He went much farther than these three: he found the nucleated cell to be not simply a common constituent, but the fundamental basis of structure. He founded his 'Zellentheorie' (1839*a*, p. 197) chiefly on his own discoveries. His contributions to the problem of cellular individuality will be mentioned under Proposition V.

In his first cytological paper (1838*a*) Schwann notes the strong resemblance to the cellular tissues of plants shown by notochordal tissue and cartilage, which he had studied in the larvae of the spade-footed 'toad', *Pelobates fuscus*. He mentions the nuclei of both kinds of cells, each containing one or more 'Kernkörperchen'. He regards the embryo as cellular: 'Since therefore the serous and mucous layers of the blastoderm consist of cells and the blood corpuscles are cells, the foundation of all organs that appear later is composed of cells.' He mentions ganglion and pigment-cells and describes the cellular nature of the lens of the eye and of cancerous growths. This paper was published in January 1838. In it Schwann refers to the forthcoming article by Schleiden (the 'Beiträge'), and claims that the latter's statements about the way in which plant cells multiply are applicable also to animals.

Schwann begins his next paper (1838*b*) by stressing the importance of the nucleus in showing the correspondence between animal and plant cells. Most of the observations reported here were made on pig embryos. He records the cellular nature of horny matter, of the lining of the amnion and allantois, the surface of the chorion, the alveoli for the teeth, and the surface of tooth-pulp: all consist of cells with nuclei. He is puzzled, naturally enough, by striated muscle. He finds nuclei in the cells of the kidney, salivary and

lachrymal glands, liver, and pancreas. He notes the nucleated cells in connective tissue and considers the white fibres as projections from them. He records the cellular nature of feathers. 'So the whole animal body, like that of plants,' he remarks, 'is thus composed of cells and does not differ fundamentally in its structure from plant tissue.' In his third contribution on this subject, published in April of the same year (1838c), he deals with the cellular structure of cartilage, and shows that nail, fat, and unstriated muscle consist of or develop from nucleated cells. The paper ends with an appendix by J. Müller, in which the existence of nucleated cells in pathological growths (osteosarcoma, &c.) is recorded. The valid factual part of Schwann's great book, which was published in the following year (1839a), consists largely of a reiteration of what he had already made known in these three papers. It contains, however, a considerable amount of interesting theoretical matter, which will be discussed at the appropriate places in future parts of this series of papers.

Meanwhile, Purkinje, Henle, and Valentin had continued to make discoveries in the same field. Purkinje (1838a) mentions the 'Centralkern' in the 'Körnchen' (cells) of the liver. He also gives (1838b) an excellent figure of nerve-cells from the black substance of the cerebral peduncle of man. This is reproduced here (Text-fig. 3) so as to give a visual impression of what others were doing in the year in which Schleiden and Schwann made their results known. The nucleus, nucleolus, and pigment are well seen in the figure. Henle followed up his book (1837) with a paper (1838) in which he gave a detailed description of the cellular nature of the epithelia of the human body, including even the lining of the blood-vessels. He is, of course, familiar with the nucleus and the nucleolus; the former he here calls the 'Kern' and the latter, unfortunately, the 'Nucleus'. A useful reminder of the state of knowledge at the time of Schleiden's and Schwann's contributions is provided by the fact that Henle's paper occurs before Schleiden's 'Beiträge' in the same volume of the journal. About the same time Valentin (1838) contributed a curious paper on the differentiation of the cells of the human embryo into their definitive forms. He tried to follow the behaviour of the nuclei (Zellenkerne) during differentiation. What he writes on this subject contains much error, but he was striking out on an important new line and at least he made it clear that the nucleus is far less liable to modification during differentiation than the rest of the cell. Next year Valentin made an unequivocal statement of the correspondence of the nucleated cells of plants



TEXT-FIG. 3. Purkinje's figure of nerve-cells, published in the same year as Schleiden's and Schwann's first publications on cells. The cells are from the black substance of the cerebral peduncle of man. (Purkinje, 1838b, Fig. 16 on Plate opposite p. 174.)

and animals (1839a, p. 133). He remarks that Schwann has completed the comparison between them.

Valentin now introduced the word 'Nucleolus', in the course of an abstract of Schwann's book (Valentin, 1839b, p. 277). He does not mention that he is coining a word, but simply says, 'In mammals the cartilage-corpuscles appear to constitute the whole cell and as such to contain nucleus and nucleolus.' (Turner (1890a, p. 11) is wrong in saying that Schwann introduced the word.)

The story of the nucleus may be rounded off by mention of Nägeli's demonstration (1844) that this organelle is a characteristic component of the cells not of phanerogams only, but of all kinds of plants from algae upwards. Thenceforth there was seldom any difficulty in recognizing nuclei; appeal was made especially to the nucleolus as a distinguishing feature in cases of doubt. The use of stains in microtechnique was repeatedly rediscovered during the years 1848-58, as I have told elsewhere (Baker, 1943), and this naturally gave a great impetus to the study of what is usually the most stainable object in cells. Huxley's attempt (1853) to discredit the nucleus is therefore all the more extraordinary. He tells us (p. 297) that Schleiden's belief in the existence of nuclei in all young tissues is 'most certainly incorrect'. The nucleus, he says (p. 298), 'has precisely the same composition as the primordial utricle.' Little attention, however, appears to have been paid to him. From the forties onwards the position of the nucleus in cytology was secure: it was regarded as an essential constituent of the cell. It is to be noted that this conclusion was reached long before there was any general agreement that protoplasm was also a necessary constituent. This may at first seem strange; but it must be recollected that the nucleus is obviously easier to *recognize* than protoplasm, on account of its having morphological as well as physical and chemical characters.

The discussion of Proposition II will be continued in Part III of this series of papers. I thank Prof. A. C. Hardy for his valuable criticism of the typescript of this paper, and Miss O. Wilkinson for conscientious clerical assistance.

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